PHYSIOLOGICAL AND CYTOLOGICAL STUDIES OF THE STATOLITH APPARATUS IN PLANTS

by

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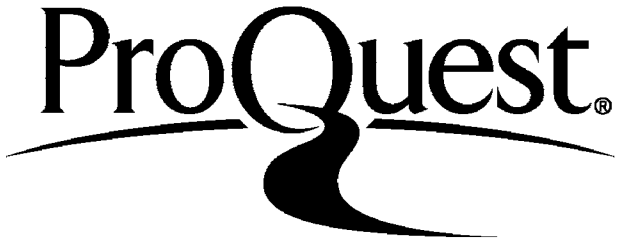

ABSTRACT

Sedimentation of starch in geoperceptive cells of plants was observed more than sixty years ago and proposed as the mechanism whereby changes in gravitational stimulus are perceived. The idea was that there is an interaction between the sedimented starch grains and the protoplasm along the lower walls of the sensitive cells, which might lead to the synthesis or release of an enzyme. It has been suggested that the highest sensitivity resides in the cytoplasm along the outer tangential walls of the horizontal root. The lower part of a horizontally placed root would thus acquire a higher concentration of the enzyme than the upper and thereby perhaps a higher concentration of a specific growth inhibitor.

Experiments have shown that the sedimentation behaviour of statolith starch under different conditions fits in well with the geotropic responses of roots under those conditions. However, the statolith starch theory of geoperception, although substantiated by much supporting evidence, still remains unproven.

Since the statolith starch theory was proposed, other cell organelles have been suggested as the perceivers of gravitational stimulus. Perhaps the most acceptable are the mitochondria. Ziegler (1953) believes that the reduction of tetrazolium salts, which he observed at the lowermost side walls of horizontal shoots, was located in mitochondria which sediment under the influence of gravity.

Hertz & Grahm (1958, 1962) incorporate Ziegler's observation and interpretation in a theory to explain the oxygen-dependent potential difference which develops between the lowermost and uppermost sides of roots and shoots during fifteen minutes of horizontal stimulation. They propose a mechanism whereby this can cause geotropic curvature.

Electron microscope observations of root-cap cells do not support the contention that the mitochondria sediment rapidly under the influence of gravity. The roots which do show significant differences between lower and upper halves of the cap-cells, have more mitochondria in the upper halves, not the lower. This difference probably results from displacement by sedimented amyloplasts. Dictyosomes show the same general pattern of distribution as mitochondria and are probably also displaced by amyloplasts as they sediment.

Němec (1901) observed densely staining lamellate and granular bodies in geoperceptive cells, which moved in relation to the direction of gravitational stimulus. He suggested that the interaction of starch movement with these bodies leads to geoperception.

With the electron microscope high concentrations of endoplasmic reticulum membranes have frequently been observed in root-cap cells in a similar position to the "lamellate bodies" of Němec, and it is considered possible that these structures are analagous. It seems probable that the endoplasmic reticulum membranes are displaced byamyloplasts as the latter sediment.

As a result of electron microscope observations of geoperceptive root-cap cells, it is considered that the amyloplasts are still the most likely organelles to trigger off the sequence of reactions leading to geotropic curvature.

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CONTENTS.

ABBREVIATIONS

"	inch
cm	centimetre(s)
mm	millimetre(s)
μ	micron(s)
$\overset{o}{A}$	$\overset{o}{A}$ngstrom(s)
ml / cc	millilitre(s)
g	gram(s)
^oC	degree(s) centigrade
$\overset{o}{...}$	degree
mV	millivolt(s)
pd.	potential difference
N KCl	normal potassium chloride
NaOH	sodium hydroxide
OsO_4	osmium tetroxide
$KMnO_4$	potassium permanganate
TNB	nitro blue tetrazolium
TNBT	2,2',5,5'-tetra-p-nitrophenyl-3,3'-(3,3'-dimethoxy-4,4'-biphenylene)-ditetrazolium chloride
Ca^{++}	calcium ions
K^+	potassium ions
w/v	expresses the concentration of a solute in terms of the number of grams of the solute in 100 ml. of the solution

CHAPTER I

INTRODUCTION

A. The statolith theory.

Generally, when a plant or animal is displaced from its normal orientation, it will respond by asymmetric growth or by movement until it has returned to its normal position in respect to gravity. In many animals such a response is known to be brought about by changes in pressure on sensitive regions of the cytoplasm. For example, certain water animals possess organs known as statocysts in which the displacement of movable "statoliths" undoubtedly gives rise to the perception of the stimulus of gravity. The idea that similar bodies exist in plants was first suggested by Berthold (1886); fourteen years later Němec and Haberlandt independently demonstrated the existence of movable starch grains*, particularly in the starch sheath of most stems, and in the tips of roots and coleoptiles. They postulated that these starch grains have a similar function to the statoliths of animals, and that the

*The movable "starch grains" are in fact amyloplasts containing one or more starch grains.

peripheral layer of cytoplasm in each cell containing movable starch grains is sensitive to the pressure of these grains; when the pressure on the cytoplasm changes because of the movement of starch grains caused by a change in gravitational stimulus, a chain of reactions occurs leading to the visible growth-response to gravity.

The statocysts of some animals are directly open to the air, and it was possible for Kreidl (1893) to replace the statoliths of shrimps with iron filings, and then to demonstrate with a magnet the statolith nature of these foreign bodies. By holding the magnet above the animal, it could be made to swim upside down. For the botanist such convincing and direct experiments are not possible, since the statocysts would have to be ruptured to introduce magnetic material. However, in the years since the statolith theory was first postulated, a considerable amount of supporting evidence has accumulated, with no convincing evidence to devaluate it, nor any satisfactory alternative theory. The supporting evidence includes

(a) the close correlation between sensitivity to geotropic stimulus and the occurrence of

statolith cells, and

(b) studies in which experimental interference with the statolith apparatus has brought with it changes in the capacity to respond to geotropic stimulation.

Generally roots which are ageotropic do not form statolith starch (Tischler, 1905), and when organs are temporarily unable to respond to gravitational stimulus, the movable starch appears at the same time as the organ develops the capacity for geotropic response. Movable starch grains occur even in members of the Liliaceae which do not otherwise form starch. The exceptions to such correlations are few: statoliths have been reported in the ageotropic secondary roots of Myosotis palustris and Oxalis acetosella (Haberlandt, 1903) and of Pyrola and Pistia (Tischler, 1905) and in the aerial roots of some epiphytic aroids (Linsbauer, 1907). There are also a few cases in which geotropic sensitivity exists without starch statoliths. For example, the rhizoids of Chara are positively geotropic and the shoots are negatively geotropic yet in neither are there movable starch grains. However, in this plant Giesenhagen (1901) observed bodies (possibly crystals) of unknown nature which moved under the influence of gravity. A few higher

plants have been reported as devoid of movable starch and capable of geotropic response. Dahlia and Allium fall into this category. It was found though during the course of this present work, that although these plants do not normally store starch elsewhere, movable starch grains are present in their root-caps. Other reports include the aerial roots of the orchid Laelia anceps (Tischler, 1905) and the perianth of Clivia nobilis (Linsbauer, 1907), which do not possess movable starch and yet do respond to gravitational stimulus. Such examples are not so easy to explain on the statolith theory, unless it be assumed that other heavy particles which have not been detected act as statoliths in these plants. Calcium oxalate crystals have been shown to behave as statoliths in the sensitive nodes of wheat plants (Prankherd, 1920), and it is possible that other heavy particles exist in Laelia anceps and Clivia nobilis as well as in the fungi and other geotropic plants without starch grains.

Besides correlation of geotropic sensitivity with presence and absence of starch, quantitative measurements of movable starch have been compared with sensitivity. Notable among such comparisons is the work of van Guttenberg (1911) who compared the behaviour of Setaria and Sorghum.

In the first species sensitivity is distributed uniformly along the whole of the coleoptile, and so are the movable starch grains. On the other hand, in Sorghum, where the upper four millimetres of the coleoptile are most sensitive, the starch grains are concentrated in the same region.

Correspondence between geotropic sensitivity and the distribution of starch grain statoliths is also shown during ageing: ageing brings about the disappearance of both sensitivity and movable starch grains. When sensitive regions persist as in the "Gelenknoten", so as a rule do these regions retain their movable starch.

Such instances of correlation between geotropic sensitivity and presence of starch could be multiplied, but the examples given illustrate well the wideness of the correlation.

The first record of experimental interference with the statolith apparatus is that of Charles and Francis Darwin (1880) who cut away the tips (1-1.5 mm) of roots and observed that no bending occurred if geotropic stimulation followed decapitation. Bateson, Darwin and Fitting (1888) then discovered that normal

geotropic curvature occurred if geotropic stimulation were applied prior to decapitation. From this they inferred that the root-cap perceives the stimulus. Such experiments have been repeated many times, but none of them allows an unambiguous conclusion to be drawn. Němec, worried lest the decapitation effect resulted from wounding rather than the removal of the statocysts, made several transverse incisions in the root-tip which collectively produced a wounded surface equal to that created by the removal of the root-cap. The reduction in geotropic response was not nearly so marked after wounding as it was after decapitation. Another experiment which shows that wounding does not prevent geotropic curvature was that of Tischler (1910) who decapitated the roots of Sonneratia which have a well-developed statolith apparatus in the cap cells. However, the geotropic reaction remained unchanged after decapitation. Tischler attributed this to the exceptional presence of a starch-sheath in these roots.

Decapitation experiments of Brauner (1923) and Anker (1955) with Avena coleoptiles and of Brauner and Hager (1958) with Helianthus hypocotyls are not so easily explained on the basis of the statolith theory, for although they illustrated complete loss of response if

they were stimulated after decapitation, they could bring about almost normal curvatures by placing stimulated, decapitated organs vertically in indol-3-acetic acid. Thus, undoubtedly the extending zone is sensitive to gravity.

Experiments designed to remove statolith starch without surgery are also ambiguous since the methods used to change the starch metabolism might also evoke other effects which might prevent the geotropic response. Such experiments include embedding roots in plaster (Němec, 1900); the starch disappeared, and so did the capacity of the plant to respond geotropically after the plaster had been removed. Gradually the starch re-appeared in the root-cap, and with it came the capacity for geotropic response.

Haberlandt (1902-1903) also found correlated loss of geotropic sensitivity with loss of starch after cold treatment, yet plants which had had prolonged cold treatment but still contained starch grains still could respond normally to gravity.

Low temperature experiments are reported in this thesis. In these, low temperatures were used to increase the viscosity of the protoplasm of the

root-cap cells. Thereby it was hoped to reduce the movement of starch and to discover whether there was any reduction in geotropic response by such treatment. In general the viscosity of living protoplasm rises as the temperature is decreased, the rise becoming rapid as 0°C is reached. (Pantin, 1923-1925; Greely, 1904; Weber, 1916; Weber and Hohenegger, 1923.) Reports have been made of organisms in which viscosity reaches a maximum at about 15°C and decreases as the temperature is raised or lowered about this point, and rises again very sharply at 0°C (Heilbrunn, 1922). Heilbrunn suggests that the viscosity of protoplasm, as opposed to a purely physical system, can undergo "autoregulation" by the protoplasm itself. Weber has measured the change in viscosity of the starch sheath cells of Phaseolus multiflorus, using the rate of movement of starch as a measure of relative viscosity. His results show that viscosity is doubled when the temperature changes from 10°C to 0°C. Weber did not measure the effect of low temperature on geotropic response. This has been done by Czapec (1898), Bach (1907), Hawker (1933) and Prankerd (1935). In all cases the presentation time and the latent time increased as low temperatures were approached. None of these workers went below 5°C, but Rutgers (1912) measured the

presentation time for the geotropic reaction of the coleoptile of Avena sativa using a temperature range of 0-40°C. The most rapid increase in presentation time was when the temperature was decreased from 5°C to 0°C. Apart from Hawker (1933) these earlier workers did not look at the position of the starch grains in their experimental material after stimulation at their experimental temperatures. Hawker, using a temperature range of 10°C - 40°C observed a rapid decrease in the rate of starch movement when temperatures were decreased below 30°C, and a corresponding rise in the presentation time.

The experiments recorded later in this thesis were designed to discover

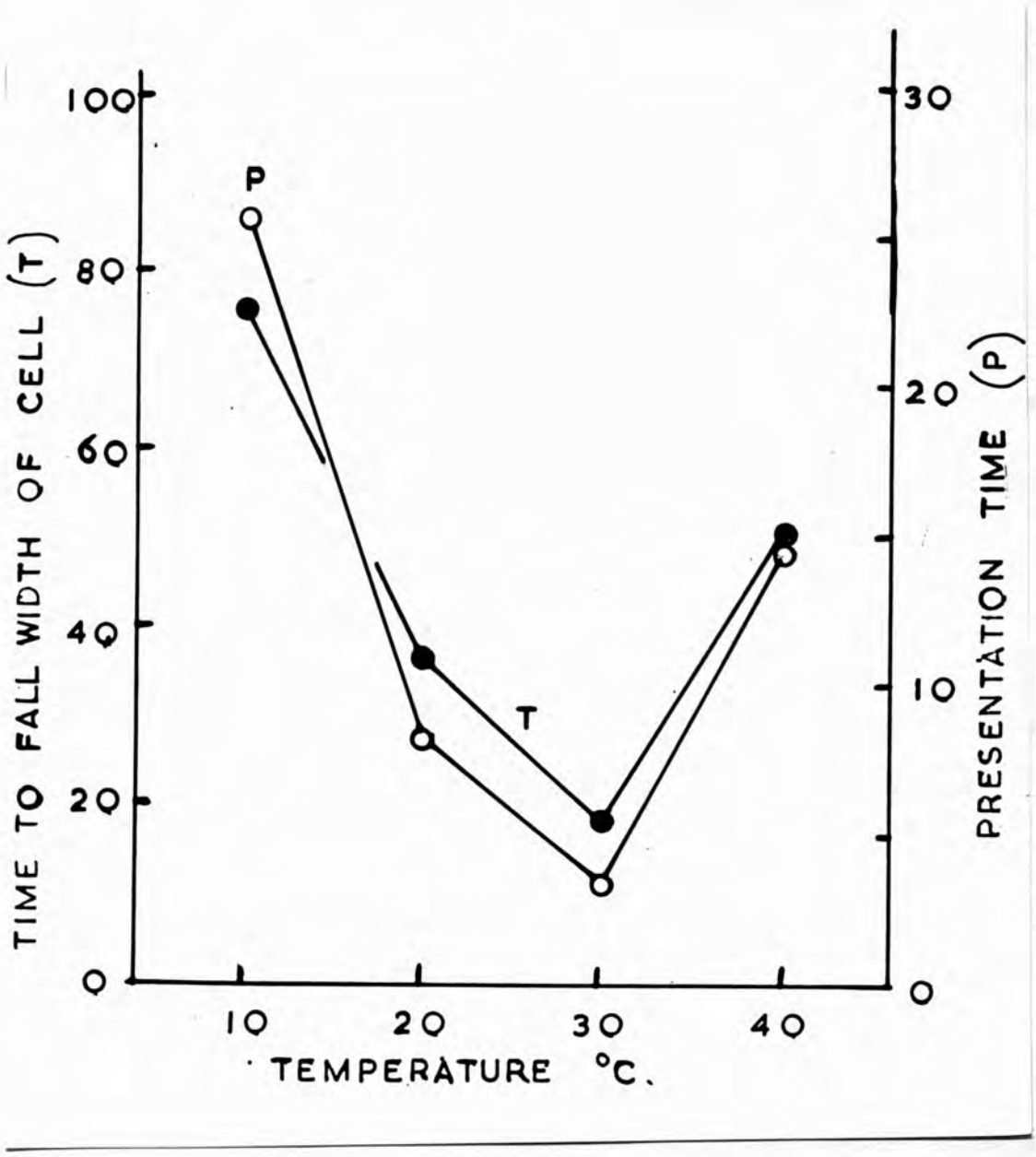
(1) whether temperature affects starch movement in root-cap cells of Vicia faba

(2) whether temperature affects curvature of these roots, and

(3) whether there is any correlation between temperature effects on starch movement and curvature.

Figure 1:

Temperature relationships of geotropic presentation time in minutes and the average time in minutes for the statolith grains to fall the width of the statocyte cell (33μ) in the seedling stem of Lathyrus odoratus. (Data of Hawker, 1933; recalculated Audus, 1962.)



Another experimental approach to the statolith theory has involved the application of centrifugal force. Reports on the behaviour of statolith starch in such experiments are not universely in support of the statolith theory. Jost (1902) found that with rotation at low speeds just sufficient to produce a curvature in response to the stimulus of centrifugal force there was NO movement of starch. The experiments of Buder (1908) on the other hand support the statolith theory but his experiments have not been confirmed. Von Ubisch (1928) could find no general parallelism between the position of the statolith starch grains and geotropic perception. The reason for such discrepancy may well be variation in fixatives. Some fixatives penetrate rapidly and prevent movement of cell organelles almost immediately after a piece of tissue is immersed in it, but others penetrate slowly. Since the early experiments of Jost, (1902) Buder, (1908) and von Ubisch, (1928) knowledge of fixatives and of their rates of penetration has increased considerably. In this thesis experiments are reported in which particular attention was paid to the speed of fixation, prior to carrying out experiments to determine the effect of rotation on the position of starch grains and to relate this to the direction of growth. Such experiments

agree with the results of Buder in that they are not in conflict with the statolith theory.

With such a wealth of physiological experiments as have been performed in the past seventy years, there is still not one which either proves or disproves the statolith theory, although the existing evidence mostly supports the theory.

An approach to which scant attention has been paid in the past is that involving cytological and histochemical techniques: Němec (1901, 1902) observed densely staining protoplasmic bodies of a lamellate and granular nature in roots which had been horizontal, fixed in chromacetic acid and stained with haematoxylin. These protoplasmic bodies always occurred at the ends of the cells where statolith starch is normally situated (i.e. prior to horizontal stimulation). They appeared when the starch grains moved from those walls under the influence of gravity, and were pushed out of position by the return of the starch grains after placing the organ vertical again. They then slowly disappeared. It was this interaction of statolith starch with the protoplasm which Němec regarded as initiating the reaction sequence leading to curvature. Ziegler (1953) made a study of roots and shoots which

had been horizontal, to determine whether gravitational stimulation affected the localisation of reduced tetrazolium salts after growing the organs on tetrazolium agar. He observed that tetrazolium salts were reduced to their red-coloured form only at the distal end of the statolith cells (i.e. at the boundary of the cytoplasm directly under the plastids) in roots which were grown vertically, whereas in roots which had been horizontal the reduced tetrazolium was localised along the lowermost side-walls (i.e. still at the boundary of the cytoplasm directly under the plastids). The red colour of the reduced tetrazolium was localised, he said, in small particles of about the size and shape of "microsomes" and he suggested that these small coloured components of the cytoplasm function as statoliths.

Apart from these experiments of Němec and Ziegler, detailed study of cell organisation in statolith cells has been neglected. Hertz believes on theoretical grounds that mitochondria would sediment fairly rapidly under the influence of gravity, and he uses Ziegler's results to support his hypothesis: but Ziegler himself stated that the particles he saw were not mitochondria, but microsomes ("Obes sich bei diesen neuen statolithen-körpern um Microsomen handelt, wie ihre

Grösse und Form wahrscheirlich macht, konnte noch nicht endgultig sichergestellt werden"). It is probable though that by the term "microsome" Ziegler meant "mitochondrion", since in a private communication to Hertz he says that they are about 1 μ in diameter and that they stain with Janus Green. Unfortunately he does not include the photographs in his paper, but in the original photographs which were lent to me for a short time, the particles were similar in size to mitochondria.

The contention of Hertz that mitochondria sediment under the action of gravity is included in a theory proposed by Cholodny (1922). He suggested that sedimentation or "creaming" of particles of about the size of mitochondria would give rise to a concentration gradient across the cell. He claimed that such particles would have a negative charge and therefore cause a potential difference across the cell. This would cause a migration of cations to maintain electro-neutrality; the monovalent ions such as K^+, being more mobile than the divalent ions such as Ca^{++} would establish differences in the monovalent/divalent ion ratios on the two sides of the cell. These differences by modifying permeability of the protoplasmic membranes

would finally induce different growth rates on the two sides of the cell. "Leaving out of consideration the plausibility of this shift in ion ratio and the elaborate theories necessary to explain positive and negative responses, the theory fails to account for the non-growing tips being the most sensitive perceptors; in other words, it fails to account for the indirect action of the tip in response." (Audus, 1962).

B. The geoelectric effect.

Cholodny's idea of the existence of a potential difference across the cell is incorporated in the main alternative theory to the statolith theory, which for the last thirty years has been favoured probably because it is possible to formulate a mechanism for linking geoperception by the development of an electric potential to the chemical control of curvature by the unilateral distribution of auxins far more easily than it is to link the movement of starch grains with unilateral auxin distribution (Audus,1962).

Bose (1907) first observed that an electric potential is produced by the influence of gravity, between the lower and upper sides of a horizontally placed shoot- or root-tip. Brauner (1927, 1928) repeated these experiments and detected a potential difference across the root after only a few seconds stimulation. This reached a maximum of 8-9 mV in about 10 minutes, the upper side of the root being negatively charged.

The experiments of Brauner were repeated by several later workers (Clark, 1937; Schrank, 1947; Jantsch, 1959; Hertz, 1960) and similar results were obtained. However, the use of liquid contact electrodes led to serious criticisms. McAulay and Scott (1954) and Wartenburg (1957) attributed the effects observed to artefacts caused by the electrodes. These criticisms were overcome by Hertz and Grahm (1962) who developed a technique in which the plants were not touched by the measuring electrodes. By the use of vibrating gold leaf electrodes Grahm and Hertz confirmed that an electric potential does develop as a result of horizontal stimulation and that the lowermost side becomes positively charged. But unlike the previous workers they found that there was a lapse of about 15 minutes after the

start of stimulation before any geoelectric potential could be detected, and they obtained convincing experimental evidence that this lag is no artefact.

In most geotropic organs the presentation time is considerably less than fifteen minutes and it seems probable that the geoelectric effect rather than leading to geoperception results from it. This is supported by experiments of Grahm and Hertz (1962) in which they studied the course of the geoelectric potential for horizontal induction times shorter than the latency period. They observed a geoelectric potential with induction times as short as two minutes, i.e. of about the same duration as the presentation time. In all these experiments the latency period remained about 15 minutes. Grahm and Hertz found that the geoelectric effect does not develop in an atmosphere of nitrogen, suggesting that it is coupled with oxidative metabolism. This also supports the contention that it is not a direct effect of gravitational stimulus and it might reflect the setting in train of differential metabolic processes leading to the curvature response.

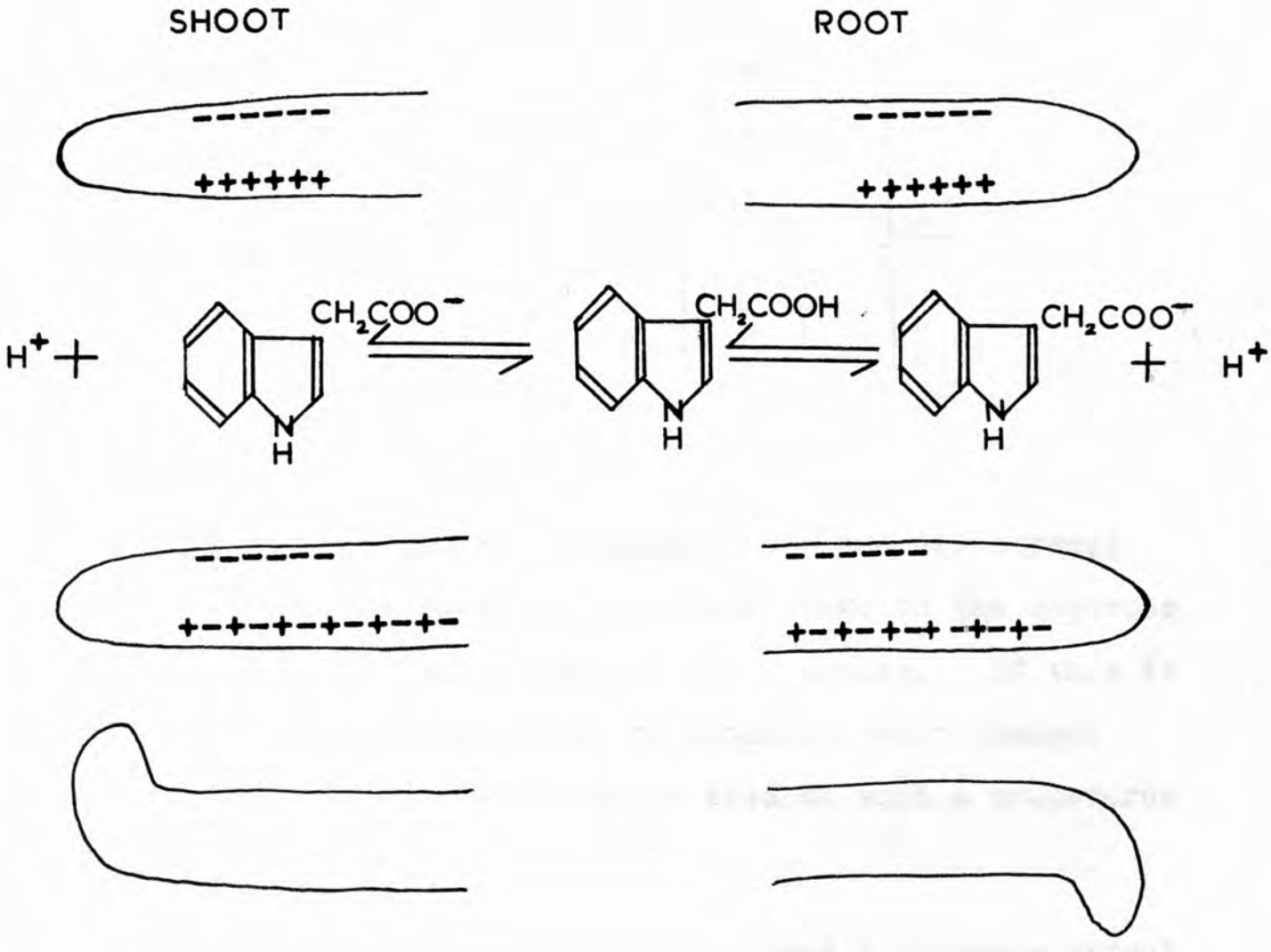
C. The linkage of perception and response.

The concept that the curvature of geotropically stimulated roots and shoots results from directed migration of auxin to the lowermost side has been a prominent feature of the theory of tropisms for the last thirty years. It has been shown by Dolk (1930, 1936), Navez and Robinson (1933), Dijkman (1934) and van der Laan (1934), that the amount of growth substances diffusing from the lower half of the horizontally placed organ is higher than the amount diffusing from the upper half. The question as to how these differences arise is still unanswered.

Although the results of Dolk and others seem to indicate that more growth substance is available at the lower side, it is possible that there is equal distribution between the upper and lower half, and that growth substance diffuses more readily from the lower half. If this is true, it is necessary to postulate a physiological difference between cells of the upper half of the root and those in the lower half when the organ is placed horizontal. This physiological difference could lead to a difference in reactivity to the growth substance or in sensitivity to it. The recent work on maize (Gillespie and Briggs, 1961) has

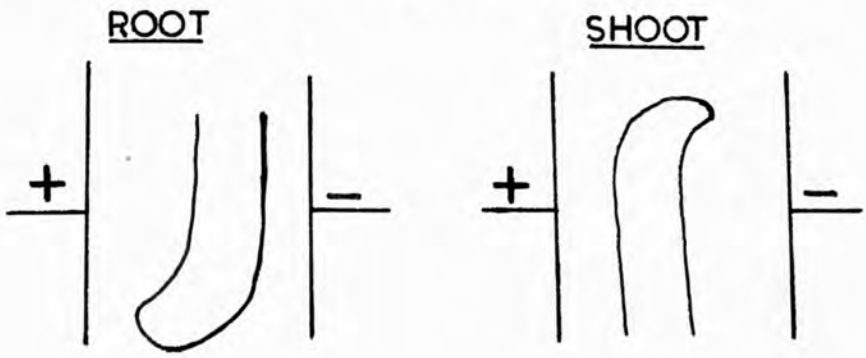
established with reasonable certainty that more auxin is released from the underside of the tip than from the upper side. There is also direct experimental evidence of a downward movement of auxin across the tip (Brauner and Appel, 1960; Gillespie and Thimann, 1961).

The downward deflection of auxin has been explained on a theory involving the potential difference which develops after the plant organ has been placed horizontal (Brauner, 1927, 1928). This theory postulates the downward deflection of the physiologically active anion of the auxin as a result of the positive charge at the lower side of the organ. The opposite behaviour of roots and shoots as a result of the increased auxin content at the lower side of the organ can be explained fairly easily: there is a supra-optimal auxin content in roots, and any increase in auxin leads to inhibition of growth. The increase at the lower side of the root will therefore lead to downward curvature. The auxin content of the shoot, on the other hand, is sub-optimal and growth is stimulated by increases in auxin. Consequently shoots curve upwards when they are placed horizontal.



It is possible that the geoelectric effect is incidental to the development of geotropic curvature. This seems unlikely though, since Bose (1907) was able to induce curvature in vertical organs by applying a potential across the organ so that one side was positive and the other negative. Consequently curvature towards

the positive plate was induced in roots, and shoots curved towards the negative plate.



So it would seem probable that the development of a p.d. represents an important stage in the sequence of events between perception and response. If this is the case, it is necessary to establish what changes gravity can cause which would lead to such a transverse polarity.

Hertz and Grahm (1958) reported a membrane effect which they claim could link statolith sedimentation with the geoelectric effect. They discovered that if they immersed an ion-selective membrane horizontally in a solution of potassium chloride and allowed a thin layer of powdered calcium carbonate to settle on the upper surface of the membrane, a potential difference developed across the membrane. With 10^{-3} N KCl solution this was as great as 20 mV. This potential is caused by

an exchange of Ca^{++} and K^{+} ions whereby the accumulation of a concentrated solution of potassium carbonate on the upper surface of the membrane gives rise to a Donnan potential over the membrane.

McNally and Stuart (1942) discovered that a similar effect occurs in the balance organs of fishes in which a calcium carbonate "otolith" leads to the development of a potential difference across the surrounding membrane which is closely surrounded by a large number of nerve endings. Changes in potential caused by changes in the orientation of the fish are received as an impulse by the nervous system of the fish. Hertz and Grahm suggest that in plant cells organic particles such as mitochondria or microsomes or salt crystals might act similarly to the otolith, if they sediment onto plant membranes causing a difference in membrane potentials. Presumably this would lead to redistribution of auxin, which in turn would lead to geotropic curvature.

It is possible on the other hand that the geo-electric effect is not an essential part of the geotropic reaction and that the curvatures observed by Bose after applying a p.d. across the plant organ are not strictly comparable with geotropic curvatures. If this is the

case the geoelectric effect in horizontal organs is incidental to the geotropic reaction, and the explanation of Hertz and Grahm linking the statolith theory with curvature by way of the geoelectric effect and auxins must be abandoned.

Instead we may try to explain the triggering off of unilateral auxin production and transverse polar auxin transport directly from the statolith sedimentation.

It has been suggested (Audus, 1962) that transverse polar movement could be of the nature of a metabolic one-way pump, set in action and determined in direction by the contact of the perceptor particles - possibly the starch statoliths - on the "sensitive surfaces" of the lateral walls of the plant organ. Mechanical pressure of heavy statolith particles on enzyme systems in the lower part of the cell could provide a metabolic gradient between that region and the upper half of the cell below it, which might well provide a mechanism for such a specific one-way pump between those two cells.

Most roots and shoots respond to gravity independently of the side from which they are stimulated. Since the cells above the horizontal axis produce less hormone than those below it under precisely the same

gravitational stimulus, one must assume that either the sedimentation of the perceptor particles is different in the upper and lower cells or they sediment in the same way but the processes they trigger off are different. In either case the organ must possess radial symmetry. If there were a difference in sedimentation behaviour a structural viscosity of the cytoplasm would be necessary to modify the behaviour of statolith particles according to their position in relation to the horizontal axis. There is no evidence from light microscope studies that statolith starch sediments differently depending on its direction of travel.

The type of radial symmetry which is most likely then, is one based on the distribution of sensitive surfaces in the graviperceptor cells. These sensitive surfaces could be restricted to the outer or to the inner tangential walls so that only the cells below or only the cells above the main axis respectively would respond to perceptor particle contact (Audus, 1962). In the case of this kind of root-tip response the mechanical pressure of the statoliths on the outer tangential protoplasmic layer in the cells below the central axis would result in the generation of more hormone either by activating an appropriate enzyme

system or by releasing previously unavailable substrates present in that layer. The net result would be an increase in the content of hormone in the cells below the main axis (Audus, 1962).

Perhaps there is a direct effect of statolith sedimentation which leads to unequal auxin production, and an indirect effect which leads to transverse polar transport by way of the generation of a potential difference (p.d.). A possible way of discovering the importance of the p.d. in geotropic response would be to apply a p.d. to a horizontally placed organ so that the p.d. which is developed naturally within the organ is exactly opposed by the applied potential. This might not give an unambiguous result but it would provide an indication of the importance. of the natural p.d. in geotropism.

If the statolith particles are the mitochondria or some other essential component of the metabolic system, then the unequal distribution itself would be expected to cause metabolic gradients laterally across the cell, thus modifying cell behaviour. On the other hand, the gravity induced accumulation of metabolically inactive particles on one side of the cell could either modify the activity of stationary metabolic particles by direct

contact or lower their effectiveness by competing for their space and forcing them to some other part of the cell. This again would cause metabolic gradients in the perception cell (Audus, 1962).

The Magnetotropic Reaction. Professor Audus has tried to check the statolith theory by attempting to make use of the diamagnetic properties of organic matter to force statolith starch to one side of the cell. This involved placing seedling roots and shoots in a strong magnetic gradient under conditions in which the effects of gravity were eliminated by rotation at an optimal speed of one revolution every 2 minutes.

Although strong growth curvatures were observed down the magnetic gradient, it was confirmed that there was no significant movement of starch statoliths associated with the curvature. Unlike the growth responses of plants to gravitational stimulus, the growth response of roots and shoots were both in the same direction in relation to the magnetic gradient, indicating that the phenomenon of magnetotropism is not comparable with geotropism. Consequently we cannot infer that the statolith theory is invalidated by this experiment.

D. The effect of statolith sedimentation on the fine structure of the cell.

The development of the electron microscope opens up new possibilities in the investigation of the effects of gravity which may well open a way to a new understanding of the mechanism of perception of gravity by plants. It should be possible by this means to discover the true nature of Němec's lamellate and granular cytoplasmic bodies, and to check the observations of Ziegler. Further, the electron microscope might reveal other unsuspected changes following geotropic stimulation. For example, small particles incapable of falling under gravity by virtue of their own mass might adhere to heavier particles which carry the smaller particles with them as they fall; redistribution of cell organelles such as mitochondria, dictyosomes, ribosomes and endoplasmic reticulum should be detectable if it occurs, with the electron microscope, providing that fixation of the cells after treatment can be achieved sufficiently rapidly and reliably.

Not only is it possible to observe the distribution of cell organelles which are below the resolution of the light microscope with the electron microscope,

but it can be used to a limited extent for the demonstration of the localisation of specific enzymes or enzyme systems.

The tetrazolium salt which Ziegler used cannot be seen in the electron microscope in either its reduced or its non-reduced form. However, it is now possible to demonstrate the site of specific dehydrogenase enzymes in plant cells in the electron microscope using nitro-blue tetrazolium or tetranitroblue tetrazolium chloride. In each case the reduced form is opaque to electrons. By this means succinic dehydrogenase has been localised in the cristae of certain mitochondria. Perhaps Ziegler's localisation of tetrazolium reduction was due to localisation of reducing activity of the enzyme rather than to localisation of the organelle containing the enzyme. Such a possibility could be checked by the use of the electron microscope and TNB or TNBT.

Chapter III in this thesis is concerned with the electron microscope observations made on unstimulated roots and roots which had been horizontal for 20 minutes. i.e. long enough for the root to have perceived but not to have responded by growth to gravitational stimulation.

An attempt has been made to analyse the distribution of organelles in electron micrographs statistically to demonstrate whether there is any re-distribution of organelles during the initial 20 minutes horizontal stimulation.

Detailed examination of the electron micrographs was made in order to discover whether gravity has any effect on the ultrastructure of the cell in other ways than by effecting the distribution of cell components.

The final chapter of the thesis discusses in the light of the preceding chapters the validity of the statolith theory and the other theories which have been propounded for the mechanism of the perception of gravity by plants.

CHAPTER II

EXPERIMENTS CORRELATING CURVATURE AND AMYLOPLAST SEDIMENTATION

A. Plant material and experimental methods.

(i) Plant material

(ii) The experimental tank

(iii) The klinostat

(iv) Recording methods

(v) Measurements

B. Preparatory techniques for light microscopy.

(i) Fixation

(ii) Embedding

(iii) Sectioning

(iv) Staining

C. Correlation of normal geotropic response with the movement of amyloplasts.

(i) Reaction time and the rate of curvature

(ii) Presentation time

(iii) The rate of movement of amyloplasts during horizontal stimulation

D. Correlation of the direction of growth of roots on a klinostat with the position of amyloplasts.

(i) The effect of rotation on the direction of growth

(ii) The effect of rotation on amyloplast distribution

E. Correlation of the effect of temperature on on stimulus perception with its effect on the movement of amyloplasts during geotropic stimulation.

(i) The effect of stimulation at low temperature on geotropic curvature

(ii) The effect of low temperature on amyloplast movement during geotropic stimulation

Figure 4: The Experimental Tank

THE EXPERIMENTAL TANK.

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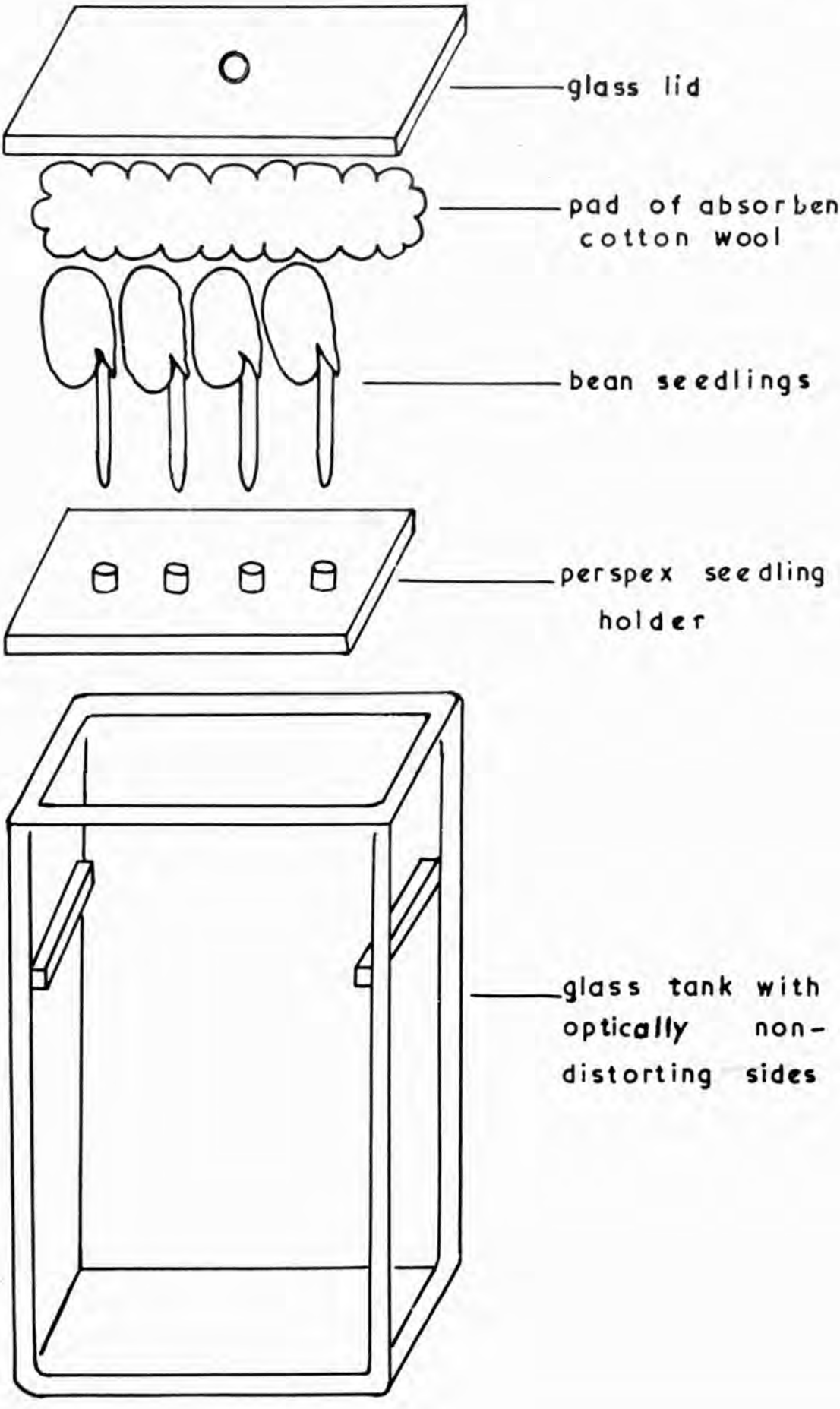


A. Plant material and methods.

(i) Plant material. Broad beans (Vicia faba, variety Leviathan) were soaked for 24 hours in running, aerated water (Audus 1956). They were then sown in moist sterilised sand in 8cm. deep earthenware pots. During sowing abnormally large, small or wrinkled seeds were discarded. The seeds were planted with their long axes vertical so that the radicles were already pointing downwards.

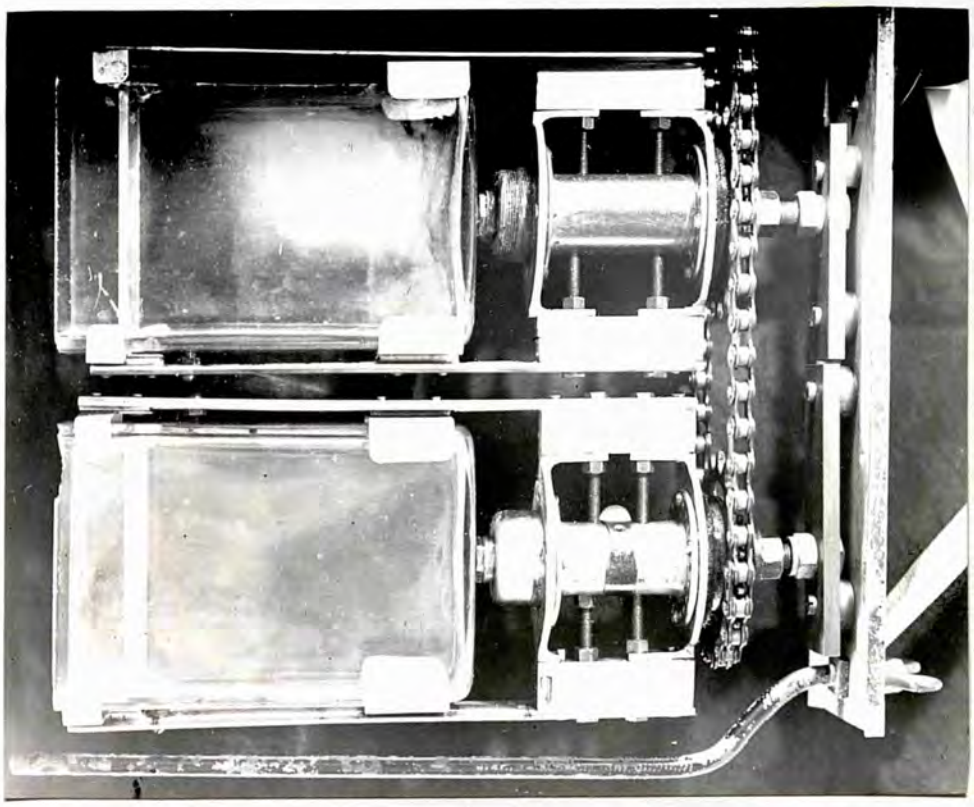
They were covered with a thin (about 5 mm.) layer of moist sand and left in a dark, saturated atmosphere at 25± 1°C. After two days' growth the seeds were carefully removed from the sand and, keeping them orientated normally with respect to gravity, they were dipped in water to free them from sand grains and transferred to the experimental tanks.

(ii) The experimental tank is shown in Figure 4. The seedlings were held firmly in position by the pad of cotton wool which was saturated with water and wedged between the cotyledons and the glass lid. The latter was sealed to the tank with vaseline. Two tanks were used in each experiment and they were arranged one above the other, each in the metal socket framework of a klinostat.

(iii) The klinostat is illustrated in Figure 5. It was first used by Audus and Brownbridge (1957).

Each metal socket framework holding an experimental tank could be rotated about a horizontal axis at the same speed by a bicycle chain and sprocket gearing. Rotation was effected by an electric Kymograph motor with its driving shaft horizontal. The whole apparatus was kept in the dark at 25± 1°C. The rotation rate could be varied as required so that one revolution could take anything from 9 seconds to 10 minutes.

Figure 5

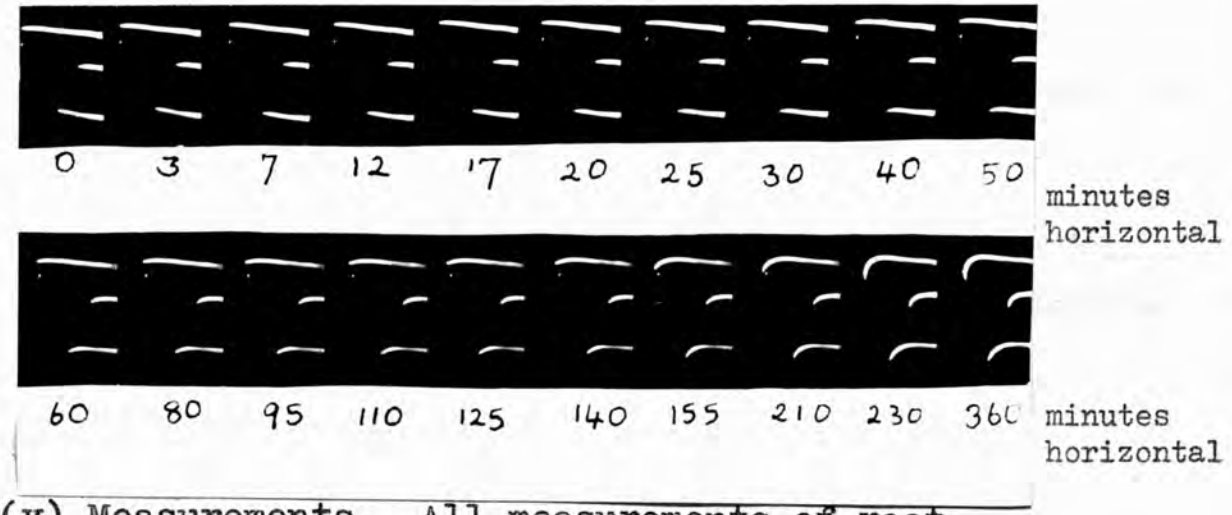


(iv) Recording methods. Photographic records showing root curvature were made using Ilford Rapid Process Panchromatic plates R.40 (9 x 11 cm.). Only that narrow vertical section of the chamber containing the two series of roots was taken. By the use of a special design of lens hood with a series of internal baffles blackened with matt blackboard paint all other parts of the plate remained completely dark during one exposure. A series of records could then be taken on one negative by arranging the camera to rotate in a horizontal plane through prescribed angles with its lens at the centre of rotation. This introduced some variation in image magnification owing to a change in lens-plate distance with angle. These were minimised by restricting the image to the centre half of the plate where angles were small. A watch was attached to a bar vertically above the roots for inclusion in the photographs. Illumination was from a laterally placed Electronic flash, Langham Press 100 Series D. It was unnecessary to stop rotation in order to make an exposure.

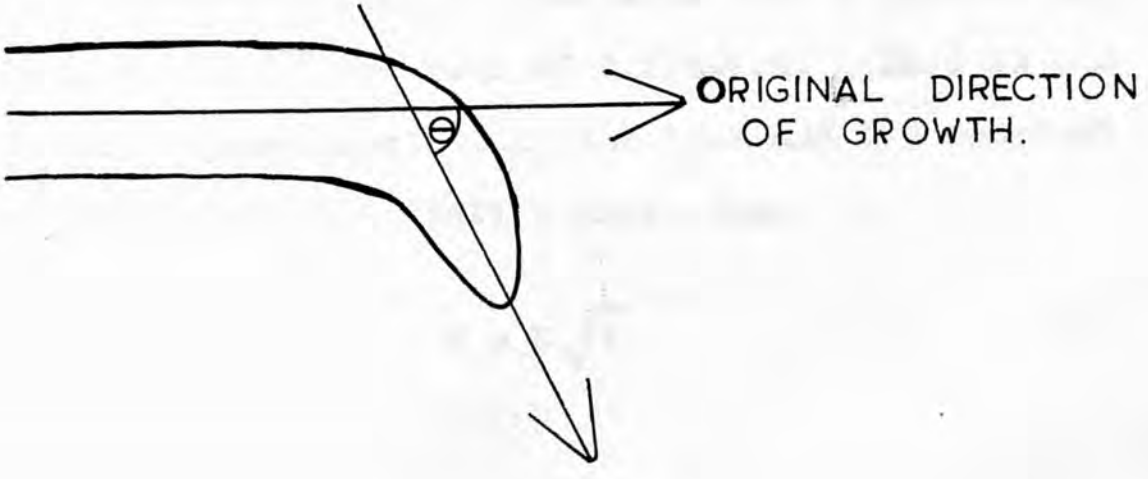
The klinostat and photographic equipment was originally used by Audus and Brownbridge and

it is described in detail in the Ph.D. thesis of M.E. Brownbridge.

FIGURE 6: Photographs of three roots to show time-course curvature



(v) Measurements. All measurements of root curvature were made from the photographic plates using a petrological microscope with a rotating stage calibrated in degrees. Angles between the directions of the tip and the main axis were measured. By this method differences in curvature of one degree could be detected.



B. Techniques used for fixing, embedding, sectioning and staining material for light microscopy.

(i) Fixation and selection of fixative.

Although preliminary qualitative studies of the root-cap cells of Vicia faba were made using hand cut sections of living material observed with a phase-contrast microscope, killing and fixation of the material was necessary for making a quantitative study of the positions of the cell constituents after various different treatments.

Because of the need to maintain the cytoplasmic constituents in exactly the same position as they were at the time of immersion in the fixative, a rapidly penetrating fixative which causes the minimum disturbance of the protoplasmic organization and minimum distortion of the arrangement of cells in the tissue had to be selected.

Medawar (1941) has shown that in general the rate of penetration of fixatives into a protein gel is governed by the laws of diffusion. If d is the distance penetrated, t is the time and K a constant depending on the fixative used, then

$$d = K\sqrt{t}$$

The only substance which has been found not to show a general obedience to this equation is osmium tetroxide. The fixed material in this case appears to offer some resistance to penetration.

Medawar's values for K are shown below. The value K represents the distance in millimetres penetrated in one hour.

Fixative	K
hydrochloric acid	4.65
nitric acid	4.3
formaldehyde	3.6
acetic acid	2.75
mercuric chloride	2.2
methanol	1.45
chromium trioxide	1.0
osmium tetroxide	0.85
picric acid	0.8

Baker (1944) has shown by using lobes of liver that the same principles govern the penetration of fixatives into tissues. The rate of entry is, however,

considerably slower, probably because the lipids of the cell-membranes act as barriers to penetration.

Of these fixatives it was decided to try formaldehyde since it has a reasonably high value of K and causes relatively little shrinkage of cells and gives better preservation of cytoplasmic detail than any other primary fixative except osmium tetroxide (Baker, 1944). This was not selected because of its slow rate of penetration and because the improvement in fixation was not so great as to warrant the expense incurred in using it as a routine fixative.

The addition of an indifferent salt to the fixative has been reported to give improved fixation in many cases. For example the addition of 1% calcium chloride to 4% formaldehyde considerably improves the fixation by reducing shrinkage, (Baker, 1944).

Chromium trioxide frequently occurs in fixative mixtures, and it was decided to try a fixative known as Craf III which contains chromium trioxide, acetic acid and formaldehyde.

One other fixative was used: this was "Zenker-without-Acetic". This is a good fixative for cytoplasmic inclusions.

Each of the five selected fixatives were tested for quality of fixation and speed of penetration. The rate of entry was determined by observing the amount of starch-grain movement which occurred during fixation. If the normal orientation of the root was maintained during fixation the movable starch grains in the root cap cells remained in a clump against the lower walls of the cells. If, instead of maintaining the normal orientation, the roots were turned through 180° about a horizontal plane so that they were upside down, and immersed immediately in a slowly penetrating fixative, movement of starch grains from their normal position could be detected, especially in the innermost cells of the tissue. If the fixative penetrated rapidly, movement of the starch-grains was prevented by immersion in the fixative. The starch-grains therefore remained in a clump against the morphologically lower walls of the cells.

The quality of fixation was assessed by comparison of the fixed, embedded and microtomed sections with hand-cut sections of living material. Phase contrase microscopy was used for making this comparison.

FIXATIVE		QUALITY OF FIXATION	STARCH MOVEMENT
I Formaldehyde		Granulation and shrinkage of cytoplasm, otherwise good preservation	Not detectable
Formalin (40% w/v aqueous formaldehyde)	10 ml.		
Calcium chloride	1 g.		
Water to make up	100 ml.		
II Clarke's		Good preservation	Detectable (10%) movement
Absolute ethanol	100 ml.		
Acetic acid (glacial)	15 ml.		
III F.A.A.		Not so well preserved as with fixatives I & II. Protoplasm was very granular.	Not detectable
Formalin	100 ml.		
Acetic acid (glacial)	50 ml.		
70% ethanol	850 ml.		
IV F.A.A. & Calcium Chloride		Not so well preserved as with fixatives I & II.	Not detectable
F.A.A.	100 ml.		
Calcium chloride	2 g.		
V Craf III		Cytoplasmic detail not well preserved	Not detectable
1% chromic acid	30 ml.		
10% acetic acid	20 ml.		
Formalin	10 ml.		
Water	40 ml.		
VI Zenker-without-Acetic		Good preservation of cytoplasmic detail	Considerable (at least 50%) movement
Mercuric chloride	5 g.		
Potassium dichromate	25 g.		
Sodium sulphate	1 g.		
Water	100 ml.		

Because of its rapid penetration combined with quite good fixation it was decided to use formaldehyde (Fixative I) for all subsequent routine fixations for light microscopy.

Half-an-hour was found to be sufficient for fixation; but no harm was detected when the tissue was left in fixative overnight.

(ii) Dehydrating and embedding. In order that serial sections could be cut in the desired plane, the tissue was dehydrated and embedded in paraffin wax.

The solutions for dehydrating were made from 95% grain ethanol diluted with distilled water.

The tissue was "cleared" with a graded series of ethanol/xylol mixtures, and infiltrated with and embedded in paraffin wax. The wax selected was Gurr's, with a melting point of 54°C, since this gave uniform blocks which were easy to cut.

(iii) Sectioning. The block was trimmed and mounted on the microtome so that the long axis of the root was parallel to the knife during cutting. All sections were cut at 6 micron.

The wax was dissolved from the sections on the slides by immersing the slides in coplin jars containing xylol. The slides were then brought step-by-step through a series of xylol/ethanol solutions of decreasing xylol content. They were then immersed in pure ethanol for 2 minutes. Subsequently they were passed through a series of ethanol/water mixtures of gradually decreasing ethanol content. Finally they were immersed in water.

(iv) Staining the sections. The most satisfactory stain for starch is iodine dissolved in potassium iodide. The formula for this is below:

Iodine	1 gram
Potassium iodide	2 grams
Water up to	300 cc.

Using this, starch-grains become blue-black, the nucleus becomes orange, the nucleolus becomes yellow and the cytoplasm yellow-brown.

Various other stains were used in conjunction with iodine to increase the contrast between starch grains and other constituents of the cell, so that minimum eye strain was experienced. It was found that staining with Fast Green after staining with iodine was a good combination. It resulted in blue-black starch grains, pale green cytoplasm and darker green cell walls and nucleus. In general though the sections were stained with iodine solution only, since this was satisfactory. Two minutes in the iodine solution was sufficiently long for staining.

Storage of sections after staining. It is difficult to make permanent preparations of iodine-stained sections and the most convenient way of storing the sections was found to be immersing them on the slides in the stain in Coplin jars.

C. Correlation of normal geotropic response with movement of amyloplasts.

(i) Reaction time and the rate of curvature.

There is always a lag between the application of gravitational stimulus and visible response to it by curvature. The duration of this lag is known as the Reaction Time. The following experiment was devised in order to discover the reaction time and subsequent course of curvature of primary roots of Vicia faba.

METHOD: Seeds of Vicia faba were soaked for 24 hours and grown for 2 days in sand. Seedlings with straight roots 2-3 cm. long were selected and put in the experimental tanks. They were covered with wet absorbent cotton wool and left for three hours to recover from their change in environment. The tanks were then turned through 90° and inserted in the metal socket framework of the klinostat. The roots were then horizontal. They were left horizontal for 8 hours during which time they were photographed at intervals. The curvature of the roots was measured from the photographs.

Figure 8: Table showing time course of geotropic curvature

Minutes Horizontal	Total curvature for each of ten roots										Mean Curvature
	I	II	III	IV	V	VI	VII	VIII	IX	X	
0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	1	0	5	0	0	0	0.6
10	0	0	0	3	2	0	2	0	6	0	1.3
15	0	0	0	2	5	-2	1	0	6	0	1.2
20	1	-3	0	2	5	-2	1	0	6	0	1.0
25	1	-2	4	5	6	-1	3	4	4	3	2.7
30	1	-3	-2	7	3	-3	3	7	6	7	2.6
35	2	-4	-2	5	4	-3	3	7	10	6	2.8
40	2	-2	-1	7	4	0	1	8	8	11	3.8
50	8	0	0	10	6	0	4	9	13	17	6.7
60	11	3	4	12	7	4	4	11	22	20	9.8
75	16	15	11	16	11	6	4	11	23	18	13.1
90	18	25	15	15	21	14	13	13	26	24	18.4
105	19	33	17	30	25	11	12	15	30	25	21.7
120	19	32	18	32	19	9	13	13	31	27	21.3
135	20	38	19	37	25	13	6	14	34	28	23.4
150	25	48	20	52	37	20	5	17	34	33	29.1
165	28	57	29	58	33	22	12	21	32	37	32.9
225	38	83	42	65	43	40	32	47	46	40	47.6
240	35	84	39	64	41	43	47	62	61	61	53.7
360	28	83	50	52	67	70	49	90	68	70	62.7
390	39	77	75	60	88	84	54	90	90	82	73.9
420	48	92	89	89	82	91	89	91	91	90	85.2
480	70	93	91	90	89	90	92	89	90	90	88.4

Negative values represent upward curvature with respect to gravity.

The course of geotropic reaction is illustrated graphically by Figure 9 and in the Table of Figure 8.

The most sensitive roots started to respond by visible curvature within the first 10 minutes of stimulation. After 40 minutes most roots had attained their maximum rate of curvature. After 8 hours most of them were growing vertically downwards.

Although selection was exercised for uniformity of samples both at the time of sowing the seeds after soaking, and at the time of transferring seedlings to the experimental tanks, the scatter of points was wide. Because of this a large number of samples was used for all subsequent treatments.

The two major parameters of the response that have been measured are the reaction time and the initial rate of curvature. A characteristic of the response is that the rate of curvature remains remarkably constant during most of the reaction, but there is some evidence that while the relationship between curvature and time can reasonably be considered to be a straight line one,

there is evidence in, for instance, roots II, IV and VIII that the curve is sigmoid. Over the main part of its surface however, it is probably a very good approximation to a straight line.

In order to obtain as objective an estimate as possible of this rate, a regression line of curvature on time was fitted to all the results. The slope of the line gave the rate of curvature, while its point of intersection with the time axis, gave an estimate for the value of the reaction time.

The correlation coefficient was $r = +0.994$ giving the following regression:

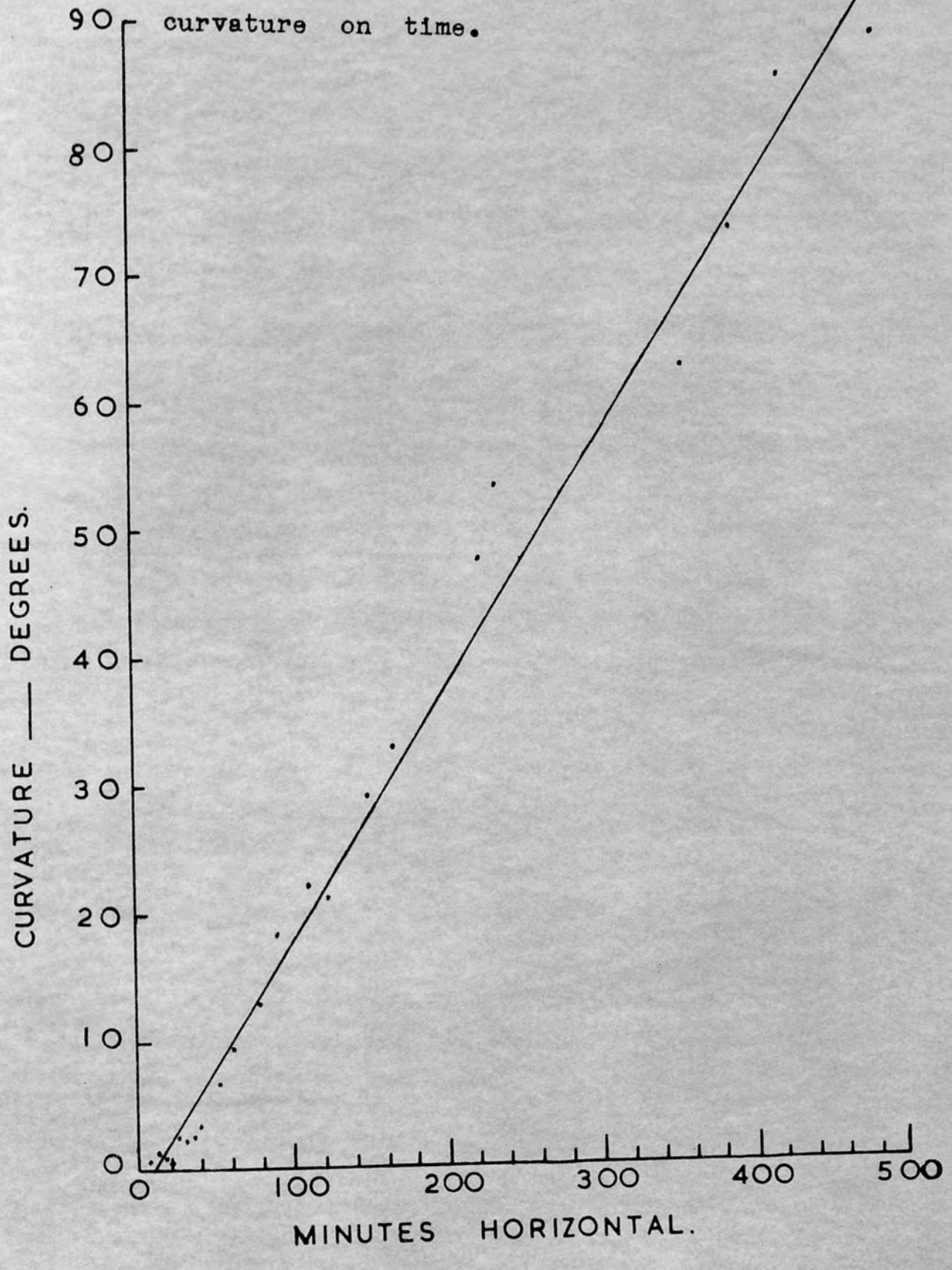
curvature = 0.196 x (time in minutes) - 1.359

From these the following figures were deduced:

Rate of curvature	0.196 degrees/minute
Reaction time estimated from the intercept of the regression line with the 0 curvature axis	6.933 minutes

During the Reaction Time the initial perception of a change in gravitational stimulus is translated, probably through a sequence of phases, to a change

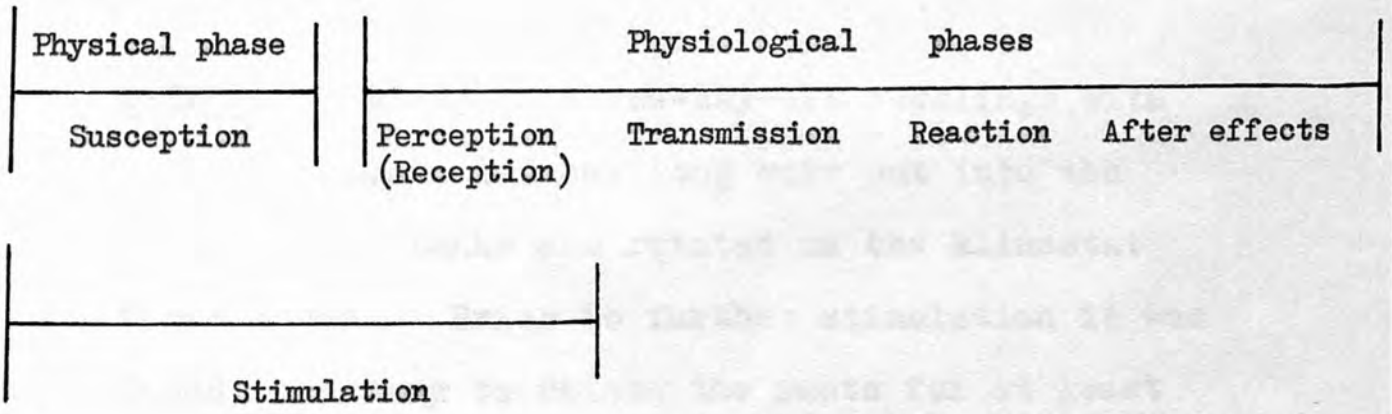
Figure 9 : Time course of curvature of horizontal roots. The mean values of IO readings are shown with the regression line of curvature on time.



in the physiological state of the root leading to the unequal growth of the top and bottom sides of the root. According to Larson (1963) the following phases can be distinguished in the reaction chain:

(a) at least one physical phase, which he calls susception

(b) several physiological phases. The first of these he calls perception or reception. During this phase a certain excitation is created. At a later stage, a phase of transmission of this 'excitation' to the site of reaction can frequently be distinguished. Still later comes the reaction proper, for instance geotropic bending, and the reaction may be followed by after-effects:



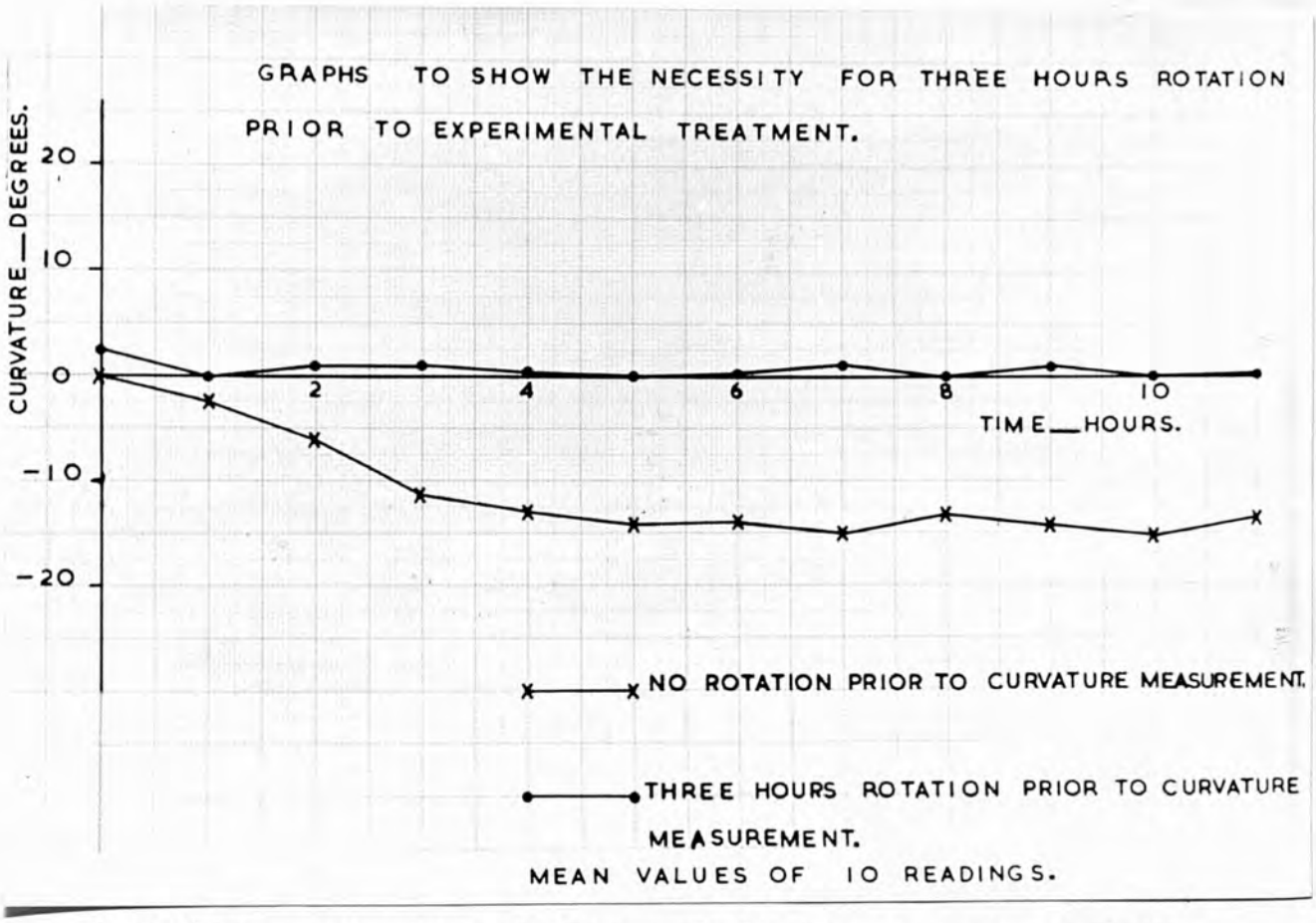
Not only are the events occurring between susception and curvature separated by a latent period, but the visible response to the stimulus is removed in space from the seat of perception. It is with the initial response which triggers off the chain of reactions that this work is primarily concerned.

(ii) The determination of presentation time.

If a root is returned from a horizontal position before curvature can be detected it will curve nevertheless providing that the period of displacement exceeds a certain minimum. This minimum period is known as the Presentation Time. The following experiment was designed to determine the presentation time for roots of Vicia faba.

METHOD: Straight, three-day-old seedlings with roots 2-3 cm. long were put into the experimental tanks and rotated on the klinostat for 3 hours. Prior to further stimulation it was found necessary to rotate the roots for at least 3 hours after transferance of the seedlings to the experimental tanks. Otherwise the small amount of

stimulation received during transferance and during the insertion of the tanks into the klinostat caused curvature. After 3 hours of rotation these changes no longer occurred. This is illustrated by Figure 10.

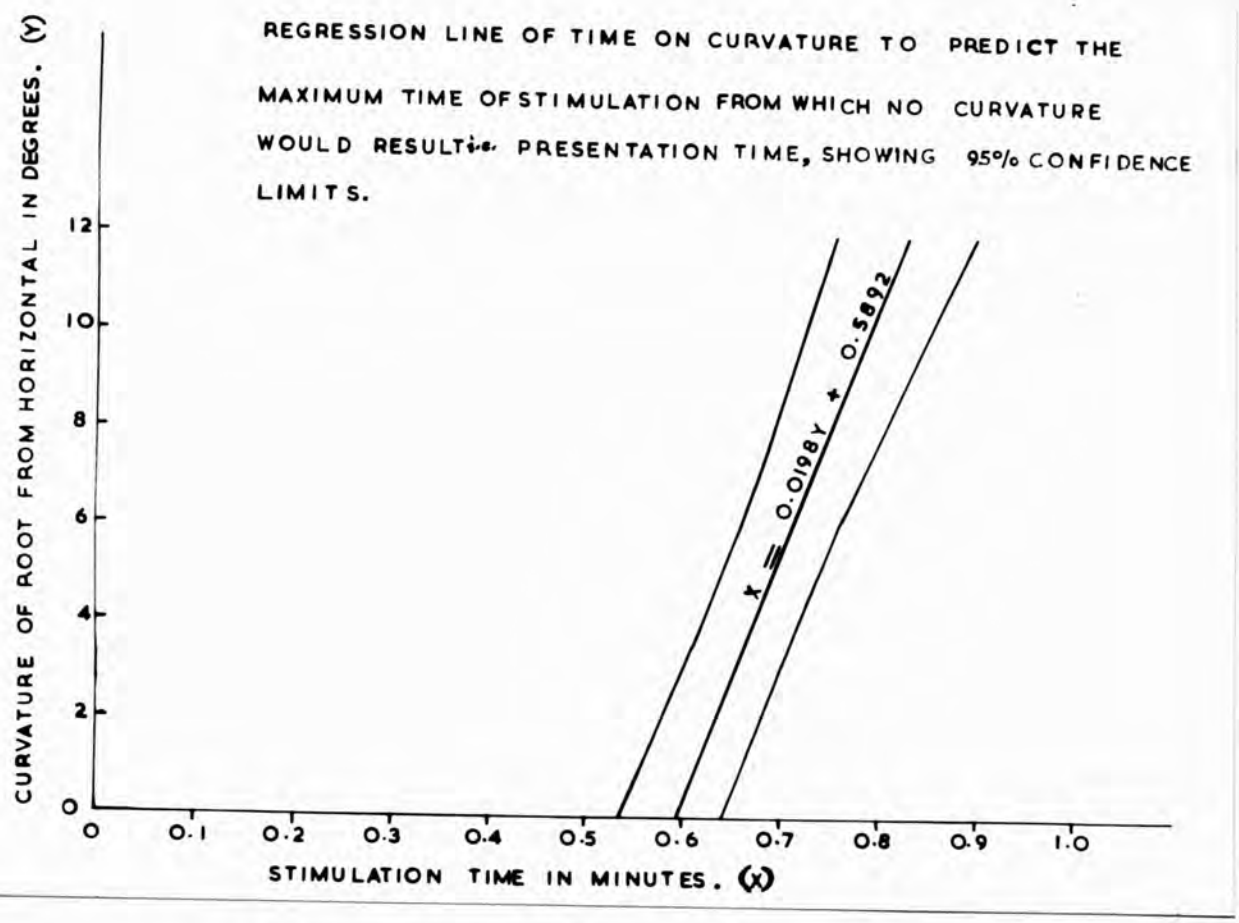


Rotation was stopped for a definite length of time and then continued. Photographs of the roots were taken at hourly intervals for 4 hours and the curvature of the roots was measured from the photographic plates. This was repeated for roots which had been subjected to 0, 1, 3, 5, 10 and 15 minutes of horizontal stimulation.

Because of the wide range of results for each stimulation time a large number of results was used in deducing the correlation coefficient for stimulation time and curvature. The correlation coefficient was found to be +0.377 which, although small, is significantly greater than zero at the 0.001 probability level.

Since the correlation coefficient proved to be significantly greater than zero it is possible to use the data to predict values of curvature from known values of stimulation time, or to predict values of stimulation time from known values of curvature. The presentation time is the length of stimulation time necessary to cause a detectable curvature. An estimate of this can be found by plotting a regression line of time on curvature. From this we can get the best estimate of the longest stimulation time from which no curvature will result. This regression line is shown in Figure 11 with 95% confidence levels on either side of the regression line. The intercept of the regression line with the 0 curvature axis gives the best estimate for the presentation time.

FIGURE 11



This is not the classic method for determining presentation time; the classic method is to find the stimulation time required for the development of visible curvatures in 50% of the plants used in the experiment. It is normal to fix a certain degree of curvature that must be reached before the organ is considered geotropically curved. Larson (1957, 1959) has criticized this method. In experiments with Artemesia roots he found that small curvatures were formed even in unstimulated roots. The curvatures were up to five degrees, positive or negative, and Larson states that if a

sufficiently fine observation technique is used, 50% curvature can always be recorded. Because of this criticism it was decided to determine the mean curvatures produced by different stimulation times and from these to predict, by extrapolation, the minimum time necessary to cause curvature. This should give the best value of the presentation time, and it is therefore a suitable method for discovering whether the presentation time is longer than the time necessary for the movement of starch grains as would be necessary on the basis of the statolith theory.

From the results of this experiment with confidence limits of 95% the range of possible values for the longest stimulation time from which no curvature ensued was found to be 0.54 to 0.64 the mean value being 0.59 minutes (see Figure 11).

Assuming that this estimate of the presentation time is approximately correct, the initial change or changes which set in train an irreversible sequence of reactions leading to curvature must occur during the first 36 seconds of stimulation. For example, if starch grain movement caused by a change in gravitational stimulus does have a rôle in graviperception,

one would expect to find that it occurs to some extent during the first few seconds of stimulation, (i.e. within the presentation time).

A second method was used to obtain an estimate of the presentation from the same data. This method involved calculating the standard deviation of the curvature measurements for each of the stimulation times. This gives a value for stimulation time above which the curvature differs significantly from zero. The results are shown below. They are also represented graphically in Figure 12.

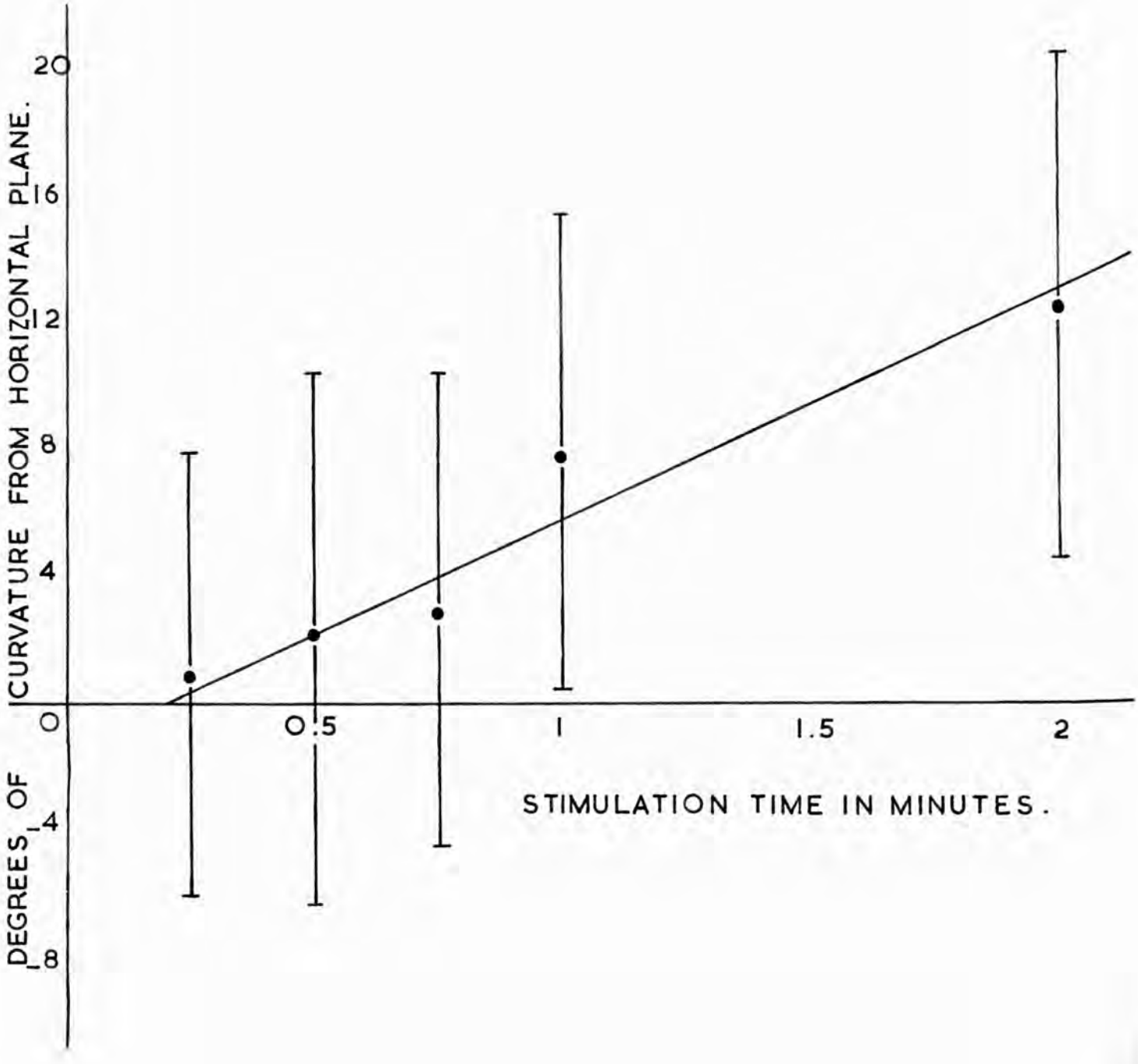
Stimulation Time (Minutes)	Mean Curvature ± Standard Deviation (Degrees)
0.25	0.86 ± 7.01
0.50	2.07 ± 8.50
0.75	2.91 ± 7.47
1.00	7.91 ± 7.60
2.00	12.50 ± 8.01

From this table it will be seen that curvature after 0.75 minutes of stimulation is not significantly larger than 0, but that after 1.00 minutes of stimulation the curvature is significant.

Table for prediction of curvature (y) at known time (x) showing 95% confidence limits.

x_o (time)	Upper limit	yo (curvature)	Lower limit
0	0.3159	-1.3980	-3.1119
0.1	0.8535	- .6801	-2.2137
0.2	1.3250	0.0378	-1.2495
0.25	1.6840	0.3967	- .8906
0.3	1.9681	0.7556	- .4569
0.4	2.5584	1.4735	+ .3886
0.5	3.1810	2.1914	1 .2018
0.6	3.8462	2.9093	1.9724
0.7	4.5611	3.6272	2.6933
0.75	4.9391	3.9391	3.0331
0.8	5.3273	4.3450	3.3627
0.9	6.1376	5.0629	3.9882
1.00	6.9816	5.7808	4.5800
1.25	9.1795	7.5755	5.9715
1.50	11.4492	9.3702	7.2912
1.75	13.7468	11.1649	8.5830
2.00	16.0620	12.9596	9.8572

FIGURE 12: Graph illustrating curvature of roots four hours after stimulation periods of different duration. The fiducial limits (2 x standard error) are shown. The regression line of curvature on time is also shown



The regression line of curvature on time is shown in Figure 12 . The regression equation is curvature = (7.177 x time in minutes)-1.3980. This line can be used for predicting the curvature at a known time. After 0.3 minutes of stimulation 95% of the roots would be expected to curve within the range of -0.46 and +1.97 degrees. After 0.4 minutes of stimulation the curvature would probably be between +0.39 and +2.56 degrees. On this basis then, one would estimate the presentation time at between 0.3 and 0.4 minutes.

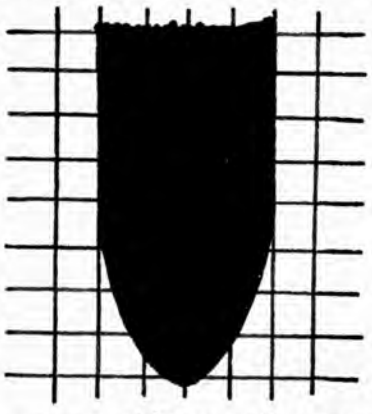
Thus by all three methods used for predicting the presentation time, it is found to be shorter than one minute. The following experiment was designed to discover how much of the starch moves within this time.

(iii) <u>The movement of amyloplasts during horizontal stimulation</u>.

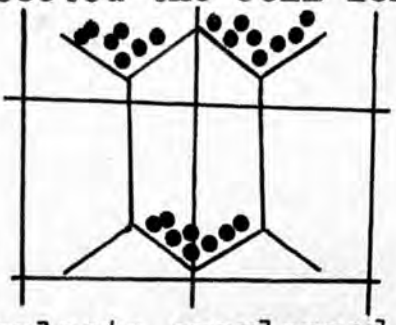
<u>Treatment of beans:</u> Seedlings with straight roots 2.5-3.5 cm. long were selected and inserted in a perspex seedling holder (Figure 13). After an hour's equilibration the beans were turned so that their long axes were horizontal. Roots were left in this position for definite amounts of

time, marked on the uppermost side with Indian ink and placed in a formalin fixing bath while still in a horizontal position. Longitudinal sections of thickness 6 micron were cut, and the distribution of starch in the root-cap cells was determined in the following way:

With a grid graticule in the eyepiece of a monocular microscope, the bean root section was orientated at low magnification so that it lay with its long and short axes corresponding with the two axes of the graticule.



The root-cap cells were then observed using a X40 objective. Counts of each cell's starch distribution were made by arranging the slide so that a line on the graticule bisected the cell longitudinally.



Some of the amyloplasts overlapped the line of the graticule which bisected the cell. In recording the distribution of such amyloplasts, the ones which were more on one side than the other were recorded as belonging to the side on which the major part of the plastid was. When it was difficult to decide to which side a plastid belonged, it was recorded as belonging to both sides.

Figure 13. Photograph of the perspex seedling holder.

Each seedling was fixed by its cotyledons on the three pins in the perspex "battlements" across the box so that the roots were pointing downwards. The perspex "battlements" could be rotated through 90° so that the roots were changed from their original normal position to a position in which the long axis of the roots was horizontal. The design of the seedling holder was such that the roots were easily accessible for rapid amputation and immediate immersion in the fixative.

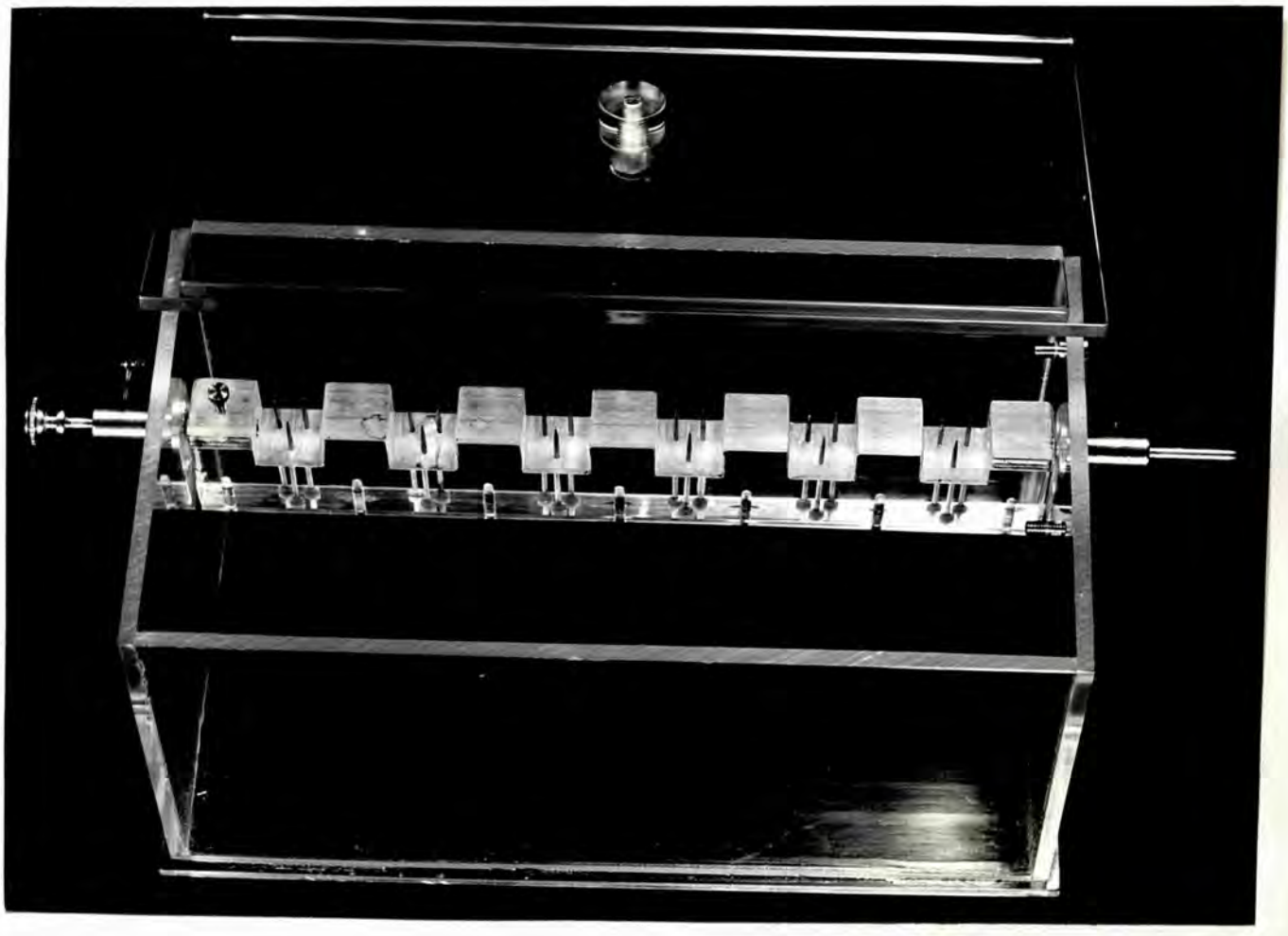
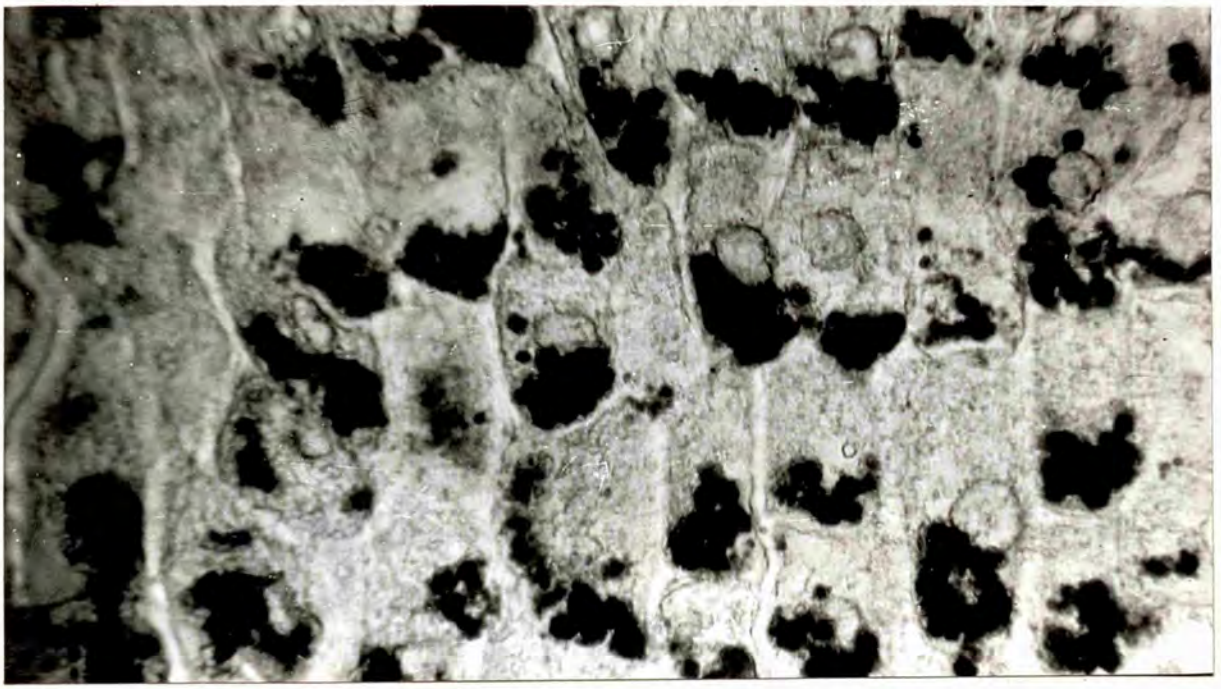


Figure 14. Photomicrograph of the root-tip of Vicia faba after 10 minutes of horizontal stimulation. The root was fixed in formalin solution, sectioned at 6 micron and stained in iodine solution. The section was cut in a median longitudinal plane.

Figure 15.

(a) Photomicrograph of root-cap cells of Vicia faba prior to horizontal stimulation. The roots were fixed in formalin solution, sectioned longitudinally at 6 micron and stained in iodine solution.



(b) Photomicrograph of root-cap cells after 10 minutes of horizontal stimulation.

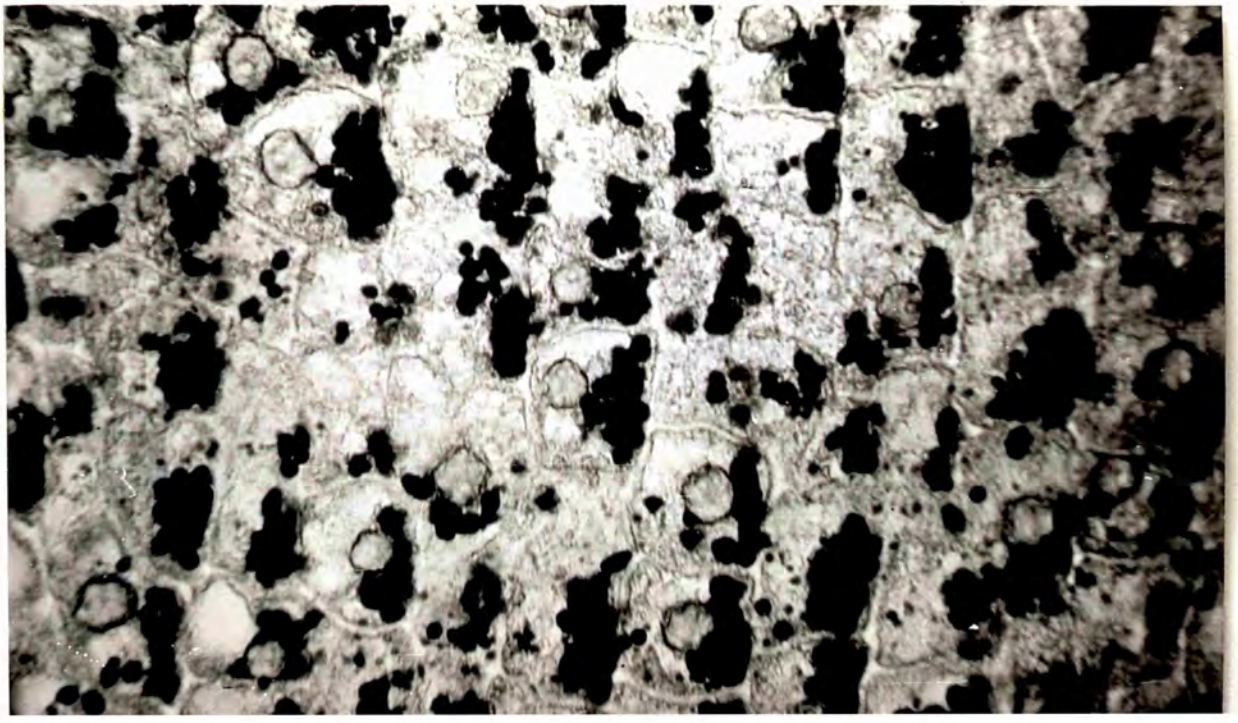


Figure 16: Diagram illustrating movement of starch in the root-cap during horizontal stimulation.

DIAGRAM TO ILLUSTRATE THE EFFECT OF HORIZONTAL STIMULATION ON LATERAL DISTRIBUTION OF STARCH IN ROOT-CAP CELLS OF VICIA FABA.

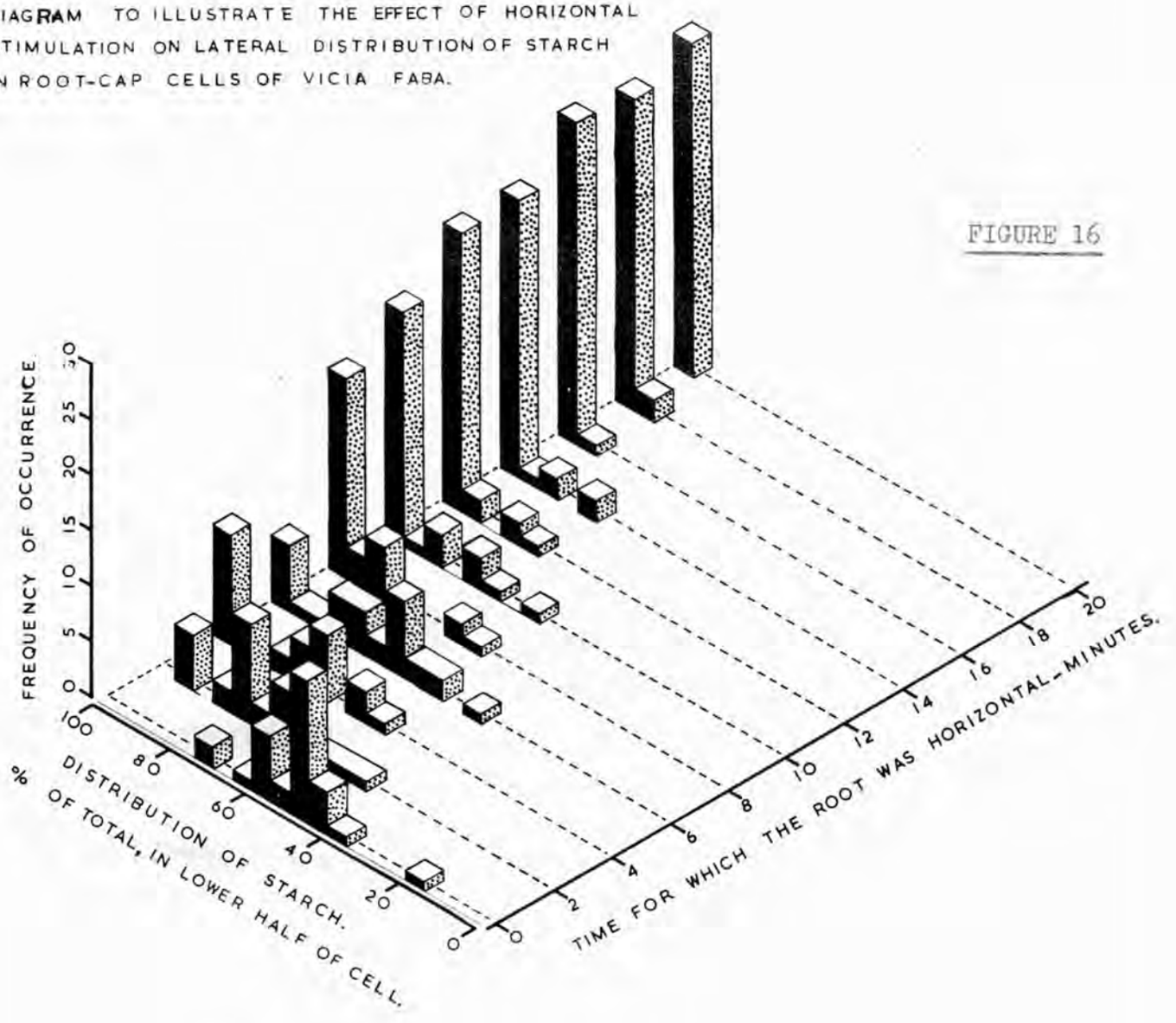


FIGURE 16

The data obtained in this experiment was used to determine how much asymmetry in starch distribution occurred during the presentation time. A chi-square test applied to the starch counts made on the two halves of root-cap cells showed that the assumption that amyloplasts are equally distributed between the two in unstimulated roots is a reasonable one. In doing a chi-square tests on the results from roots which had been horizontal for any length of time the assumption was made that the amyloplasts remained equally distributed between the two halves of the cells. If this assumption is correct, chi-square should be equal to, or less than, 6.64 at the 0.01 level of significance. The table of results is shown overleaf.

For all the counts made on horizontally stimulated roots the value of chi-square is greater than 6.64, so our hypothesis that the amyloplasts are equally distributed is not reasonable for any roots except those which have not been stimulated. Movement of amyloplasts to the lower side of the cell occurs to quite a large extent during the first 15 seconds of horizontal stimulation.

Thus it has been shown that the rate of amyloplast movement in the root-cap cells is sufficiently great to be responsible for graviperception; the time taken

Table illustrating the effect of horizontal stimulation on the position of plastids in root-cap cells of Vicia faba

Minutes of horizontal stimulation	No. of cells observed and recorded	Total number of plastids		Chi-square
		In lower half of cells	In upper half of cells	
0	60	377	359	3.54
¼	30	195	121	8.66
½	60	568	152	120.18
¾	30	215	80	30.89
1	60	474	207	52.34
1½	60	578	230	74.94
2	60	428	182	49.60
3	60	504	144	100.00
4	60	511	155	95.15
5	30	319	147	31.74
6	60	582	187	101.45
7	30	296	52	85.54
8	60	550	104	152.08
9	30	297	37	101.20
10	30	332	21	137.00
13	30	400	16	177.23
14	30	267	5	126.18
15	60	528	21	234.11

for considerable amyloplast movement to occur is less than even the shortest estimate for the presentation time, i.e. approximately 20 seconds.

Further experiments were designed to determine whether, on the basis of the statolith theory, the position of starch after rotational stimulus corresponds with the behaviour of the roots.

D. Correlation of the direction of growth of roots on a klinostat with the position of amyloplasts.

(i) The effect of rotation on the direction of growth. By rotating roots on a klinostat Larson (1957) has found that at low velocities of rotation the stimulations in all directions are sufficient for the production of curvatures and consequently the reactions follow the motion of the klinostat. At relatively high velocities irregular curvatures are formed, i.e. curvatures not induced by the gravitational stimulus. A curvature already induced will develop during subsequent rotation. At intermediate velocities the irregular curvatures are not formed and the roots remain straight. Larson concludes that "the effect of rotation on the direction of

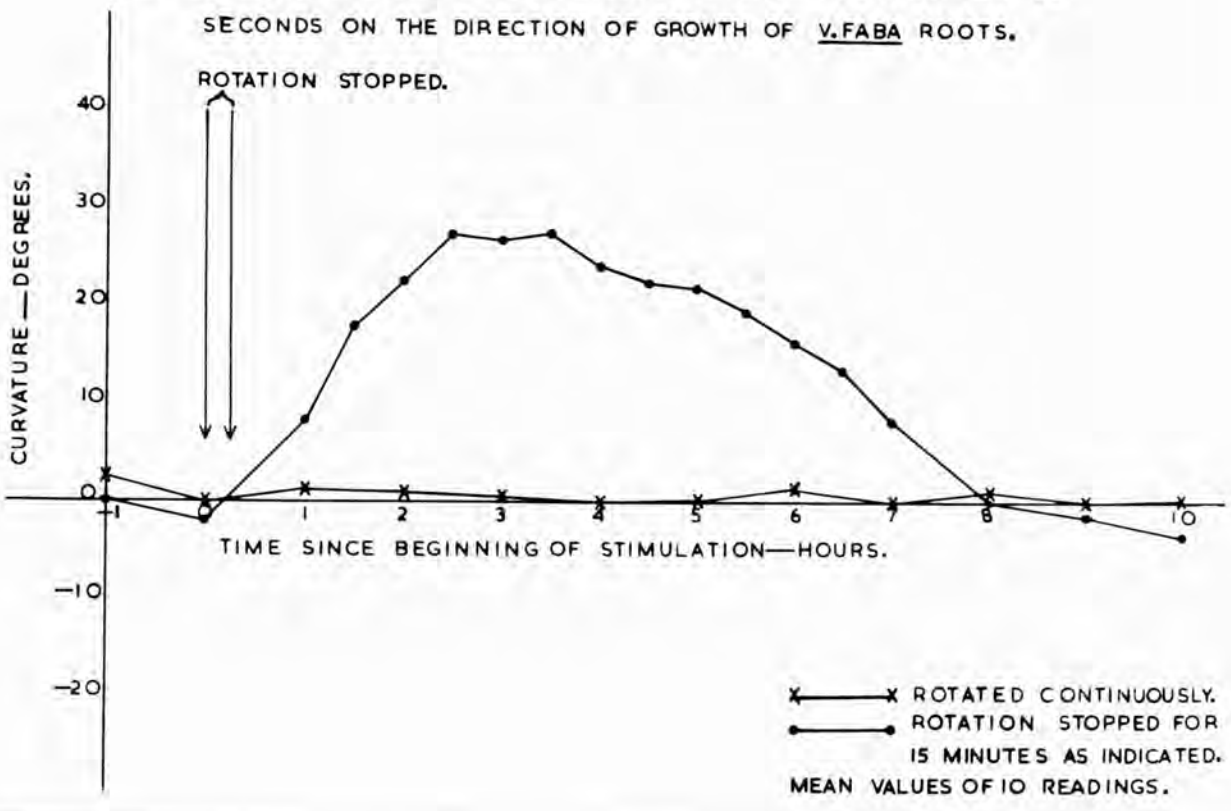
growth of roots depends on the magnitude of the individual stimuli active during one half revolution, not on their sum, which is independent of rotation velocity. The omnilateral stimulation decreases with increasing rate of rotation." Consequently, in order to allow the geotropic reaction to develop freely, i.e. without gravitational stimuli, one has to rotate the roots rather swiftly; Larson recommends one revolution each half minute. "At this speed the centrifugal force is negligible."

In the following experiments a rather slower speed of rotation was selected; each revolution took 1½ minutes. At this speed it was even safer to assume that the centrifugal force was negligible and, at the same time, the rate of rotation was sufficient to prevent induction of curvatures during rotation.

Three-day-old seedlings of Vicia faba with straight roots 2-3 cm. long were used. They were put in the experimental tanks and rotated for three hours. Rotation was then stopped for 15 minutes and then continued. Photographs of the roots were taken at regular intervals during the experiment.

When rotation is stopped for the presentation time or longer, and then continued, the root responds after 20-30 minutes to the short stationary stimulus by positive geotropic curvature, and then it reverses the direction of curvature until it lies along the axis of rotation again. This is illustrated by Figure 17.

GRAPHS TO SHOW THE EFFECT OF ROTATION AND OF HORIZONTAL STIMULATION FOLLOWED BY ROTATION AT ONE REVOLUTION PER 90 SECONDS ON THE DIRECTION OF GROWTH OF V.FABA ROOTS.



(ii) <u>The effect of rotation on amyloplast distribution.</u>

Concurrently with these experiments determining the effect of rotation on curvature, more beans were

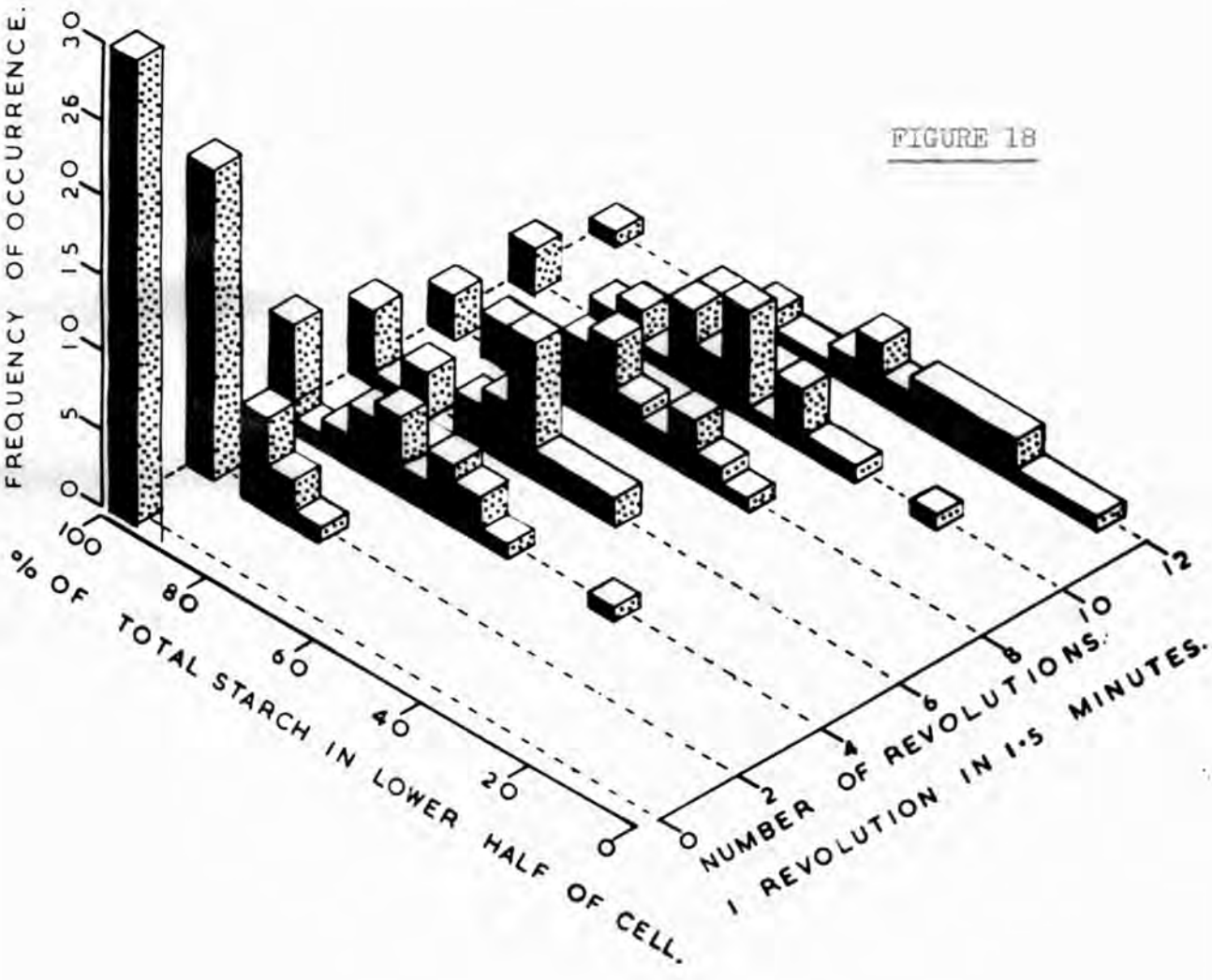
treated in a similar way and fixed after various stages in the experiment for examination of the distribution of amyloplasts. It has already been shown (see Figure 16 page 71) that all the amyloplasts of a statocyte fall to the lower side of the statocyte during the first ~~ten~~ 20 minutes of horizontal stimulation subsequent to the normal orientation of the root. Further investigation showed that during 15 minutes' horizontal stimulation following 3 hours' rotation all the amyloplasts dropped to the lower sides of the statocytes. This shows that rotation which must cause considerable movement of the amyloplasts in the cytoplasm and consequent churning of the cytoplasm has no appreciable effect on the sedimentation of the amyloplasts.

Subsequent to horizontal treatment some bean roots were rotated again and fixed after different time intervals. Amyloplast counts made on these roots illustrate that the plastids were redistributed from the unilateral position they held at the end of horizontal stimulation, until they were randomly distributed throughout the cytoplasm. This is illustrated by the diagram of Figure 18.

Figure 18: Diagram illustrating movement of starch during rotation.

DIAGRAM TO ILLUSTRATE REDISTRIBUTION OF STARCH GRAINS IN ROOT-CAP CELLS OF VICIA FABA DURING ROTATION ABOUT THE LONG AXIS OF THE ROOT SUBSEQUENT TO 20 MINUTES IN A HORIZONTAL POSITION.

FIGURE 18



These results show no discrepancy with the statolith theory since the unilateral distribution of starch grains resulting from horizontal stimulation is followed by curvature, while the subsequent redistribution of starch grains caused by further rotation is followed by growth in the direction of the axes of rotation. The time relations of these two effects are very interesting and have considerable implications. They are illustrated in Figure 19. After about 20 minutes of rotation following horizontal stimulation, the statoliths are again more or less uniformly distributed in the cells but curvature goes on for a further 2½ hours. This suggests that the stimulus once perceived is not easily reversible - it does not have to be maintained for curvature to continue. Horizontal stimulation can however be reversed by giving an opposite stimulus for the same period of time and this implies that reversal only comes about when the statoliths actually contact the opposite wall, which in turn implies that redistribution in the cytoplasm such as occurs during rotation cannot reverse the stimulus received during horizontal stimulation. These results support the implications which may be drawn from the

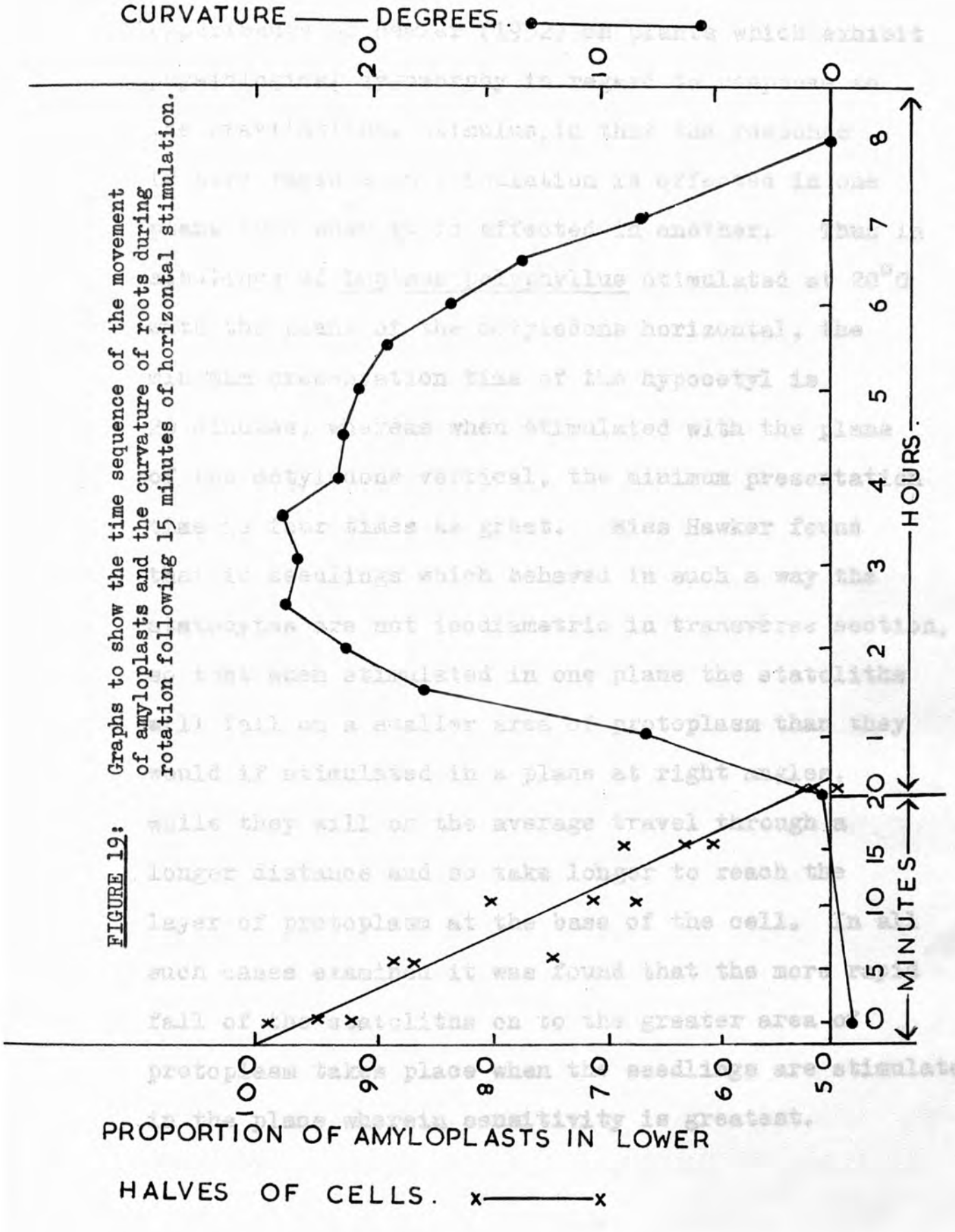


FIGURE 19: Graphs to show the time sequence of the movement of amyloplasts and the curvature of roots during rotation following 15 minutes of horizontal stimulation.

experiments of Hawker (1932) on plants which exhibit physiological zygomorphy in regard to response to the gravitational stimulus, in that the response is more rapid when stimulation is effected in one plane than when it is effected in another. Thus in seedlings of Lupinus polyphyllus stimulated at 20°C with the plane of the cotyledons horizontal, the minimum presentation time of the hypocotyl is 20 minutes, whereas when stimulated with the plane of the cotyledons vertical, the minimum presentation time is four times as great. Miss Hawker found that in seedlings which behaved in such a way the statocytes are not isodiametric in transverse section, so that when stimulated in one plane the statoliths will fall on a smaller area of protoplasm than they would if stimulated in a plane at right angles, while they will on the average travel through a longer distance and so take longer to reach the layer of protoplasm at the base of the cell. In all such cases examined it was found that the more rapid fall of the statoliths on to the greater area of protoplasm takes place when the seedlings are stimulated in the plane wherein sensitivity is greatest.

The experimental findings of von Bismarck (1959) lend further support to these implications. He found that the presentation time of Sphagnum stems is as long as 1½ hours. Correspondingly he found that amyloplast movement is remarkably slow (45 μ/hour). Thus the presentation time corresponds roughly with the amount of time taken for the statoliths to accumulate on the lowermost sides of the statocytes.

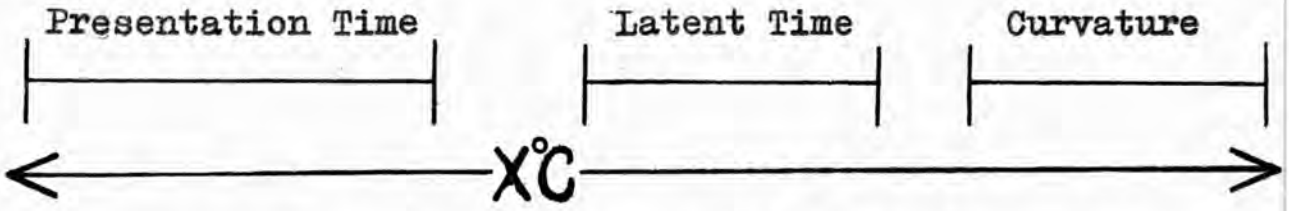
E. Correlation of the effect of temperature on the rate of movement of amyloplasts with its effect on stimulus perception.

If it is true that the movement of amyloplasts across the root-cap cells resulting from re-orientation of the root with respect to gravity, is responsible for graviperception, then the plant's ability to perceive and subsequently respond to gravitational stimuli should be effected by a change in the rate of movement of the starch grains. This rate is partly dependent on the viscosity of the cytoplasm, which in turn depends on temperature. The viscosity of protoplasm is raised in many cases by decreasing the temperature. This is the case

for instance for the cytoplasm of amoebae and other protozoa. It is also true for the cytoplasm of eggs of Nereis and for the protoplasm of the starch sheath cells of Phaseolus multiflorus. The relationship between the viscosity of cytoplasm and temperature is by no means a uniform one. For some types of protoplasm the viscosity does not decrease regularly with rise in temperature. Sometimes as the temperature is raised the viscosity may first increase and then decrease. This has been shown to be true of the cytoplasm of statocytes of Lathyrus odoratus (Hawker, 1933); between 10°C and 40°C viscosity as measured by the rate of movement of starch under gravity, decreases as the temperature is raised to 30°C; it then increases as 40°C is approached. Comparing this effect of temperature on starch movement with the effect of temperature on the presentation time for Lathyrus odoratus, Hawker found a remarkable similarity between the two effects (see Figure 1, page 17).

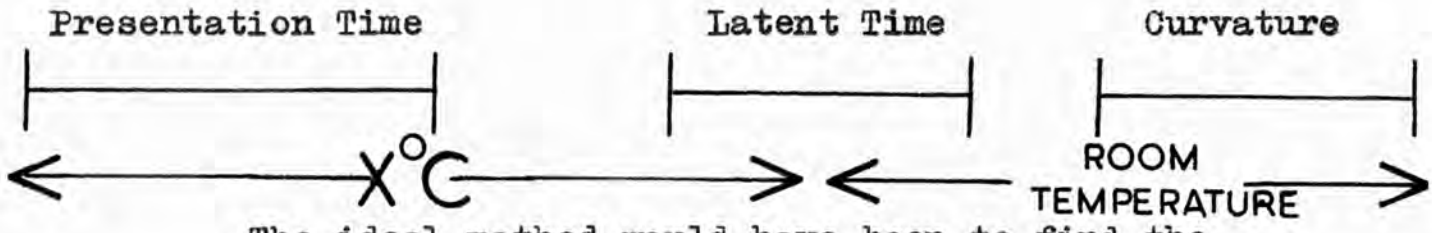
The relationship that exists between presentation time and the rate of movement of starch and between the effect of temperature on the rate of starch grain movement and the duration of presentation

time suggests that interesting results would be obtained if a study of the effect of low temperatures on the movement of amyloplasts were compared with a study of the effect of low temperature on perception of gravitational stimulus. The main difficulty in such an experiment is the isolation of the effect of temperature on the perception process from its effect on the subsequent stages of the response. Previous workers have generally found the effect of temperature on the whole of the curvature process, thus:



This method of attack does not preclude effects of temperature on various links in the chain subsequent to stimulus perception and therefore could be most misleading.

In an attempt to restrict the temperature effect as far as possible to stimulus perception the following approach was used:



The ideal method would have been to find the presentation time for stimulations at different temperatures and stimulate the roots for the presentation time at the particular temperature and then transfer the roots to room temperature and record the curvature attained. This would be a tedious method and would take a great deal of time. The results however would be of great value and interest in relation to the statolith theory.

The Effect of Temperature on the Movement of Starch.

METHOD: Temperatures of 1,2,3,4,10 and 20°C were used. Straight three-day-old roots of 2.0-3.0 cm. long were selected and put in the experimental tanks. They were left for an hour in a vertical position at the experimental temperature

before they were turned through 90° so that they were horizontal.

After 15 minutes they were fixed, dehydrated and embedded.

Longitudinal median sections 6μ thick were cut in the plane of the root which was vertical during stimulation and the distribution of starch was recorded as described previously (page 67). For each temperature the number of starch grains in the upper and lower halves respectively of 30 cells was recorded, and the standard deviation for upper and lower halves separately was calculated. The results are illustrated in the table overleaf and in Figure 20 (page 91).

This shows that stimulation at low temperatures leads to considerably less starch grain movement than stimulation at room temperature. Roots stimulated 15 minutes at 1°C do not differ significantly from roots which have not been stimulated at all.

Table illustrating the effect of temperature

on the movement of amyloplasts

TREATMENT	MEAN NUMBER OF AMYLOPLASTS		DIFFERENCE BETWEEN LOWER & UPPER
	In lower half of cell	In upper half of cell	
CONTROLS	6.03 ± 0.32	5.65 ± 0.30	0.38
15 min. horizontal at 0°C	6.16 ± 0.40	5.00 ± 0.38	1.16
15 min. horizontal at 1°C	6.42 ± 0.32	5.94 ± 0.32	0.48
15 min. horizontal at 3°C	7.59 ± 0.32	4.48 ± 0.28	3.12
15 min. horizontal at 4°C	8.08 ± 0.34	3.59 ± 0.24	4.49
15 min. horizontal at 20°C	9.18 ± 0.68	0.70 ± 0.26	8.48

The Effect of Temperature on Curvature.

METHOD: Straight roots 2.0-3.0 cm. long were selected and put in the experimental tank. They were left in a vertical position at the experimental temperature for one hour, and then the tanks were turned through 90° so that the roots were horizontal. They were left for 15 minutes and then transferred rapidly to a rotating klinostat at room temperature. They were photographed as soon as they were on the klinostat, and then at hourly intervals for 4 hours. Stimulation temperatures of 1,2,3,4,10 and 20°C were used. The mean values and the standard deviations for each of the temperatures were calculated and they are represented by Figure 21 (Page 92).

The results of this experiment show that curvature following horizontal stimulation at low temperatures is more or less prevented. At 1°C the curvature is not significantly different from the curvature exhibited by an unstimulated root. At 4°C the curvature is considerably greater, while at 10°C the curvature differs little from that of roots stimulated at 20°C.

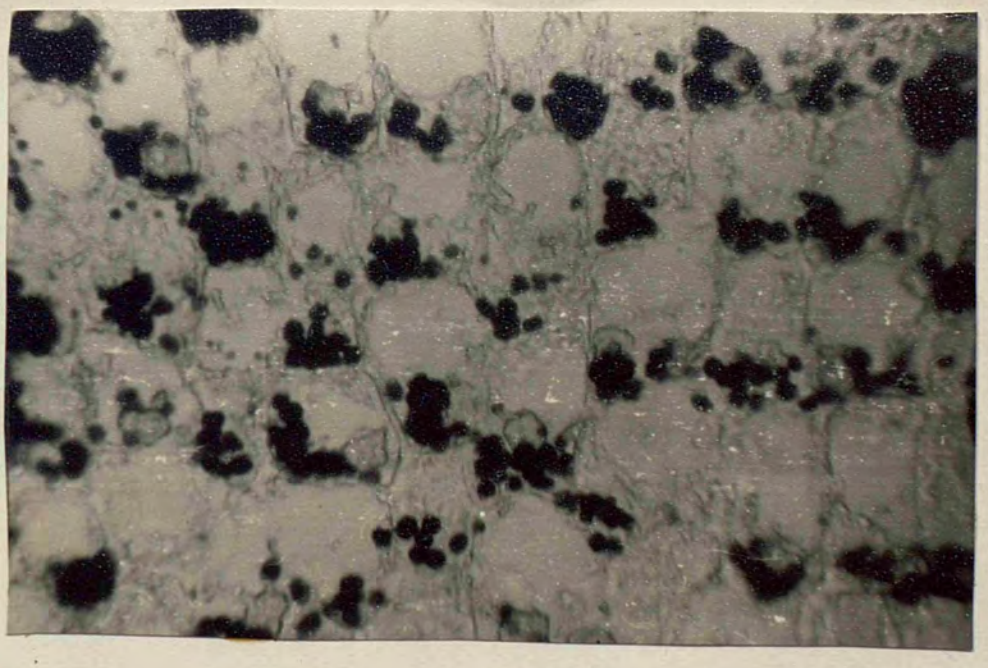
Correlation of Starch Grain Movement and Curvature over the Range of Temperatures.

It has been shown that even after 15 minutes horizontal stimulation at 1 or 2°C the roots did not curve at room temperature neither did the starch grains move. When the experiment was repeated at increasing temperatures the degree of curvature increased and, correspondingly, the proportion of starch grains which moved during stimulation, increased. (Figure 20)

The criticism applied to the experiments of previous workers that the low temperature is preventing curvature by preventing some metabolic process in the reaction chain leading to response and that the reduction of starch grain movement is incidental to, rather than functional in, the reduction of response, is far less applicable to these present results since they were obtained by low temperature treatment during stimulation, while subsequent curvature occurred at room temperature. An experiment was performed in which the roots were given short horizontal stimulation at room temperature immediately after the low temperature treatment. As a result of this, both starch grain movement and

curvature occurred, supporting the hypothesis that stimulation at low temperature affects the perception of geotropic stimulation. Further, Hawker's correlations between starch grain movement and geotropic response at different temperatures were made using presentation time as a measure of geotropic response, indicate that low temperature prevents perception rather than the transmission of, or reaction to, the horizontal stimulus.

Photomicrogaph of root-cap cells from a root which had been horizontal for 15 minutes at 1°C.



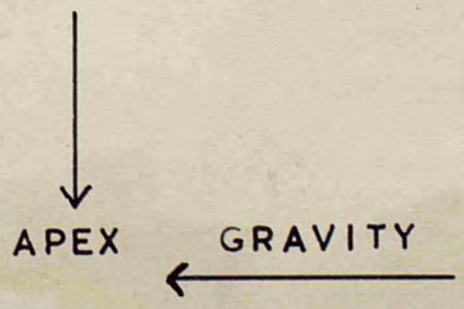


Figure 20: The effect of temperature on the movement of amyloplasts and on the curvature of roots after I5 minutes horizontal.

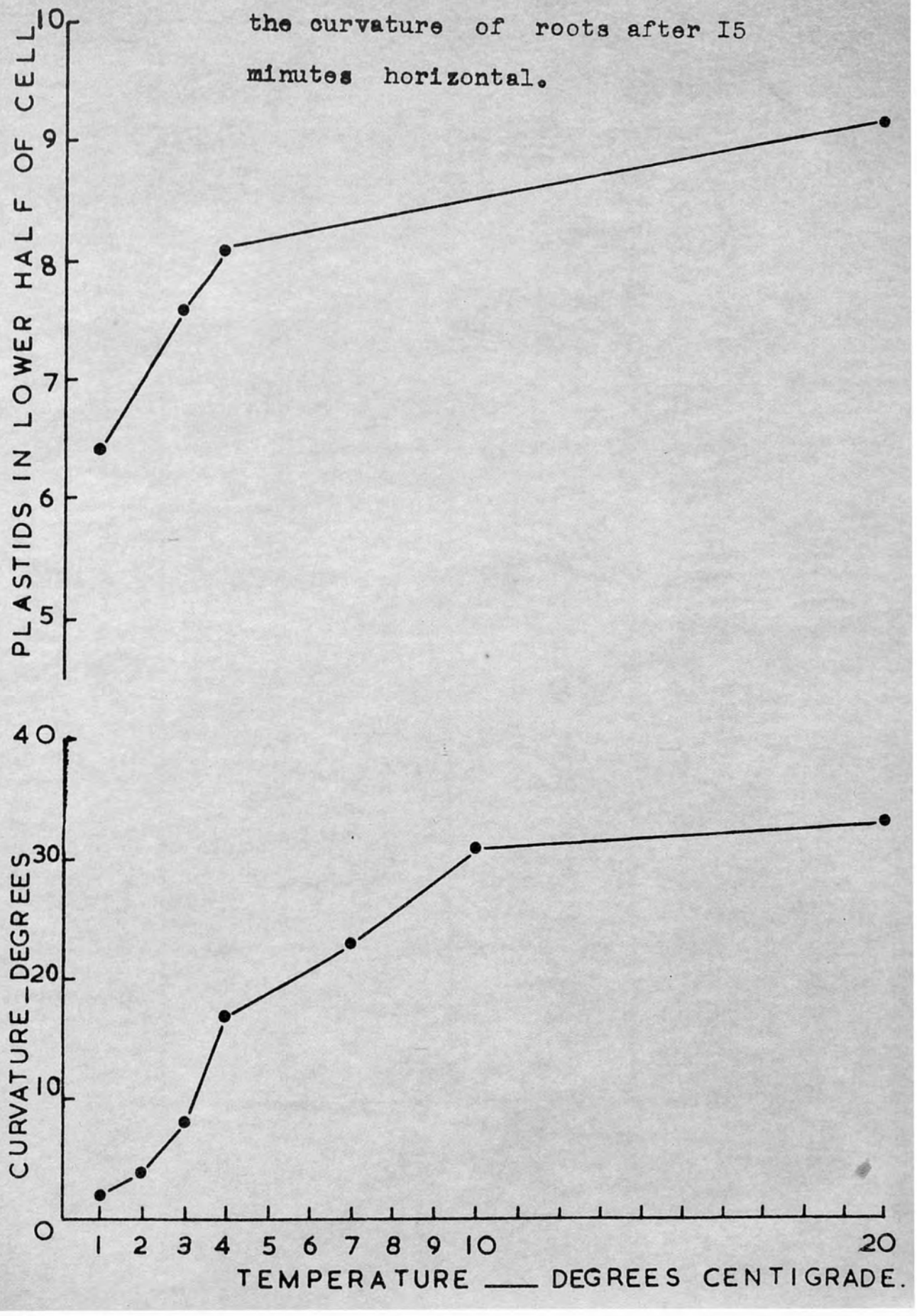


Figure 21

Temperature of Stimulation	Duration of rotation since stimulation	Mean curvature - degrees	2.S.E.
1°C	1 hour	+ .25	2.096
	2 "	- 1.38	6.301
	3 "	+ .38	6.01
	4 "	+ 2.00	5.54
2°C	1 hour	- 0.09	1.424
	2 "	+ 1.74	2.446
	3 "	+ 2.97	2.986
	4 "	+ 4.21	3.614
3°C	1 hour	+ 2.71	2.208
	2 "	+ 9.24	2.908
	3 "	+ 9.29	4.844
	4 "	+ 8.12	6.08
4°C	1 hour	2.89	1.669
	2 "	7.05	2.493
	3 "	12.18	3.417
	4 "	16.95	4.403
7°C	1 hour	5. 44	1.602
	2 "	12.11	3.865
	3 "	19.44	7.080
	4 "	22.78	9.723
10°C	1 hour	4.22	1.491
	2 "	13.07	2.704
	3 "	23.67	3.790
	4 "	30.96	5.622
20°C	1 hour	7.525	1.806
	2 "	18.53	2.933
	3 "	27.35	4.373
	4 "	32.58	6.264
Unstimulated	1 hour	-.73	1.783
	2 "	-.93	2.902
	3 "	-.80	3.336
	4 "	+.40	4.476

CHAPTER III

ELECTRON MICROSCOPE OBSERVATIONS

A. Preparative techniques for electron microscopy.

(i) Preparation of material for fixation

(ii) Fixation

(a) with potassium permanganate
(b) with osmium tetroxide solution

(iii) Dehydration

(iv) Embedding

(a) with methacrylate
(b) with Epon 812 - an epoxy resin

(v) Microtome sectioning

(a) the microtome
(b) trimming and mounting the specimen
(c) preparation of glass knives
(d) collecting and mounting the sections

(vi) Staining the sections

B. The electron microscope.

C. Photography.

D. Calibration of the electron microscope.

E. (i) Evaluation of fixation and embedding of material

(ii) Speed of penetration of the fixative

(iii) Fixation artefact caused by unilateral penetration of fixative

(iv) Conclusions regarding fixation and embedding techniques

F. The ultrastructure of the root-cap cells

G. The effect of gravitational stimulus on the ultrastructure of the root-cap cells.

(i) Redistribution of cell components

(a) method of recording distribution of cell components

(b) methods of statistical analyses

(c) results of statistical analyses

(ii) Correlation of distribution of mitochondria, dictyosomes and endoplasmic reticulum with each other

A. Preparative techniques for electron microscopy.

The techniques of fixing, dehydrating and embedding specimens in preparation for thin sectioning and electron microscopy are similar to those used in preparing sections for light microscopy, but modifications are necessary at each stage of the procedure because of the different requirements of the light and electron microscopes.

It is possible to observe much finer structures with the electron microscope than with the light microscope and these must be preserved without damage for electron microscopy. At the beginning of electron microscopy many of the techniques of light microscopy were used and it was frequently found that the specimen was damaged in a way that was not detectable using the light microscope. It is necessary therefore to establish new criteria for good fixation.

The first criterion is equally applicable to fixation for light microscopy as it is for electron microscopy. It involves a comparison of the specimen after fixation and embedding with a living specimen at the level of resolution of the phase contrast microscope. If damage to the specimen can be detected using a light microscope, it is unlikely that the

specimen will have well-preserved fine structure. Shrinkage of material can sometimes be detected with the light microscope. The dimensions and orientation of fine structures should be compatible with measurements made on living material.

A less objective approach, and one which is used most frequently for assessing the quality of fixation, is based on what the specimen ought to look like. Such a judgement includes such criteria as the continuity of membrane structures and lack of obvious distortion and discontinuity in cytoplasmic detail. While such continuity is not proof that all structures have been fixed exactly as they were in the living cell, the presence of discontinuities and empty spaces is an indication of bad embedding and/or fixation. Comparison of the results of various fixation and embedding procedures is important. Sometimes a fixative that gives poor results with one embedding medium will give good results with another embedding medium.

The range of fixatives used for preparation of material for electron microscopy is not large. Until fairly recently osmium tetroxide solution has been

accepted as the most useful fixative. It is still regarded as such for the animal tissues, but for plant material potassium permanganate is a good alternative.

Although in some features $KMnO_4$-fixed plant-cells contrast sharply with OsO_4-fixed plant cells, the basic structure is quite comparable in cells prepared by the two fixatives. This similarity serves as a valuable check on the validity of the results obtained with each of the fixatives.

Two different embedding materials were used in conjunction with the two fixation procedures. The first and most frequently used was methacrylate which is extremely convenient to use but it shrinks on polymerisation. The damage caused during shrinkage was reduced by using partially polymerised methacrylate for the final soakings and embedding (Borysko and Sapranauskas, 1954).

The other embedding material used was Epikote 812. This shrinks considerably less, but is more difficult to handle both with respect to embedding and sectioning.

Since the electron density of the various components of the plant cell is different as a result

of fixation, staining of the sections is not necessary. However, it is sometimes useful to use reagents containing metal ions which increase the contrast of the sections. For example, uranyl acetate used in conjunction with OsO_4 increases the contrast of the cell components and makes the ribosomes clearly visible.

(i) Preparation of material for fixation.

Osmium tetroxide penetrates slowly and it is important to fix as small a piece of tissue as possible so that the material is brought into contact with the fixative as soon as possible. Little more than the root-cap was fixed with this fixative.

With potassium permanganate, on the other hand, it was found possible to fix much larger pieces of tissue. This made orientation of the material easier. For example, if the root had been horizontal during treatment a portion of it could be cut away to act as a guide to the orientation of the root during treatment. A convenient shape was found to be as shown below:

To avoid re-orientation of cell components during penetration of the fixative, the same orientation of the root was maintained from the time of treatment until the end of fixation.

Wilkinson Sword edge blades were found most suitable for cutting the tissue.

(ii) Fixation.

(a) With potassium permanganate: In order to discover the optimum conditions for fixing the root-cap cells of Vicia faba with potassium permanganate, three factors were taken into account

Factor	Variations tried	Fixative which gave the least discontinuity of membranes and least disturbance of the cytoplasm
1. Concentration of $KMnO_4$	1%, 2%, 5% w/v	2%
2. Length of time of fixation	2, 15, 30 and 60 minutes	30 minutes
3. Temperature of fixation	2°C, 20°C	No detectable difference

Since there was no detectable difference between material fixed at 2°C and that fixed at 20°C it was decided to carry out subsequent fixations at 20°C, this being more convenient.

(b) With osmium tetroxide solution: With this fixative it was necessary to discover the optimum fixation conditions by varying the following factors:

1. pH
2. tonicity
3. temperature
4. OsO_4 concentration
5. length of time for fixation

Caulfield (1957) worked with plant tissue and reported that optimal preservation is obtained with 2% osmium tetroxide (w/v) solution buffered with acetate-veronal to pH 7.4. He found that there is a greater proportion of well fixed tissue in each block when sucrose is added in the quantity 0.015 g./ml. He fixed his specimens for 30 minutes at 0 - 4°C. Porter and Machado (1960) obtained good fixation of onion root-tips using the method of Caulfield. Because of its

previous success it was decided to follow this method exactly, using root-tips of Vicia faba, before any modifications were tried. As a result of this method reasonable fixation of root-cap cells was obtained, and the technique was adopted for all routine osmium-fixed preparations.

(iii) Dehydration. Before fixed tissue can be embedded in either methacrylate or in epoxy resin, dehydration is necessary. Ethyl alcohol is miscible with methacrylate monomers and with Epon 812, and a graded series of this alcohol was used for dehydration. To minimise the risk of leaching material from osmium-fixed specimens while they are being dehydrated dehydration was completed as rapidly as possible. At least in the early stages, dehydration was carried out at near zero temperatures for the same reason.

Some of the material represented in this thesis was dehydrated by passing it through a series of solutions of different alcohol content. The series generally used was 0,20,50,70,90, and 95% alcohol allowing about 5 minutes in each bath followed by 30 minutes in each of 3 changes of absolute alcohol. After some experiments it was decided that better

results were obtained with potassium-permanganate-fixed material if at the beginning and end of dehydration alcohol was added drop-by-drop so that the material was not subjected to such violent changes. Because of this improvement in results a gradient dehydration method was developed. This method eliminated sudden changes of alcohol content which were suspected of causing damage to the tissue when the step-by-step method of dehydration is used. The technique was based on the "Gradient Elution" method developed by Kellie, A.E. and Wade, A.P. (Biochem. J., (1957) 66, 199) for column chromatography. The apparatus is shown in Figure 22. It was designed and built for me by Mr. D. Burnett.

Procedure:

Fixation and the first stages of dehydration were carried out at 0 - 4°C. When the tissue to be embedded had been fixed, it was placed on the glass wool plug in the treatment chamber with enough de-ionised water to cover it. Approximately 10 mls. of de-ionised water were then run quickly from the mixing chamber through the treatment chamber. This removed excess fixative from the tissue. While there was still sufficient water in the treatment chamber to cover the tissue, tap C was closed.

Figure 22: Apparatus for dehydration.

Figure 22: Apparatus for gradient dehydration.

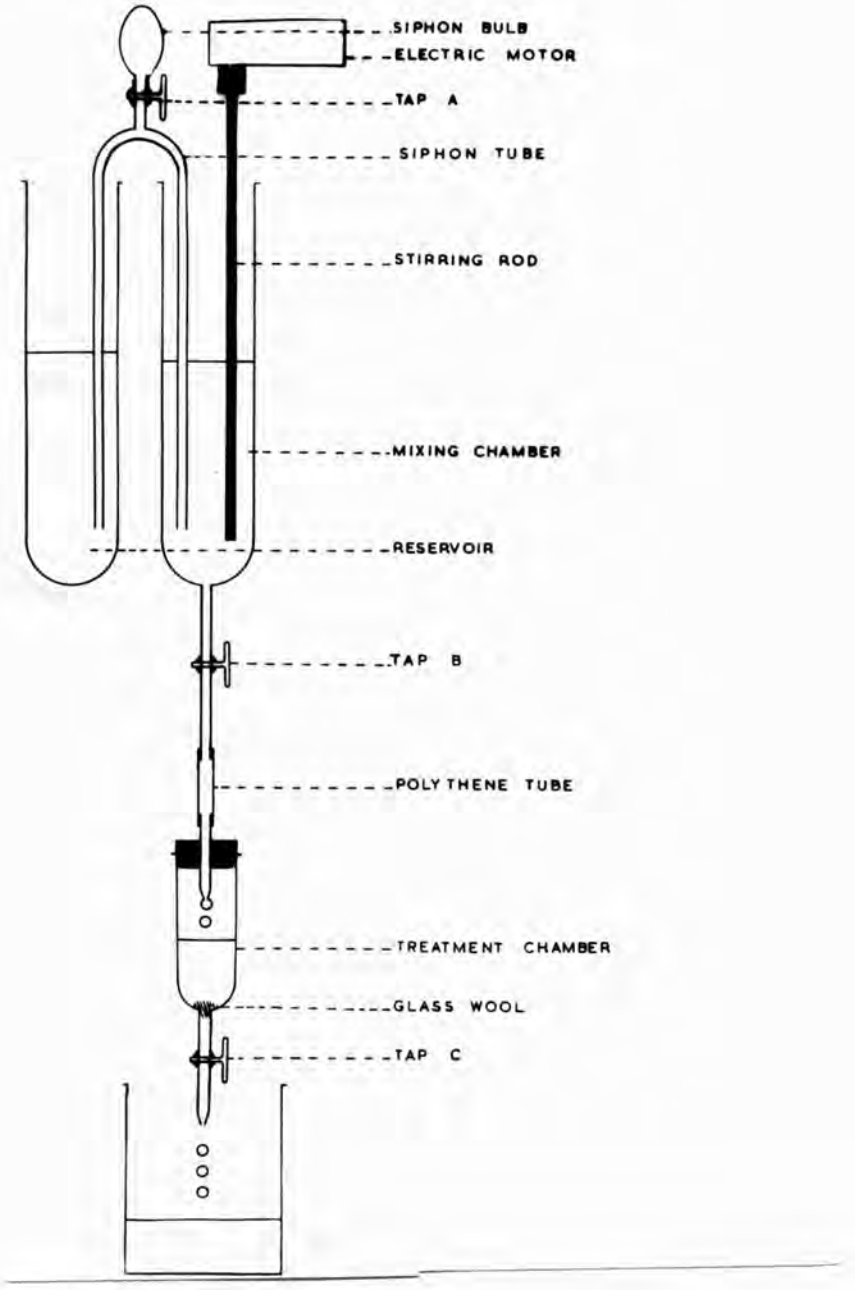


Figure 22: Apparatus for dehydration.

De-ionised water was used on the assumption that most proteins would be less soluble in this than in salt solutions, (Pease, 1960). Alternatively, for material which had been fixed with osmic acid, standard veronal buffer solution, prepared as for the fixative but with distilled water instead of osmic acid, was used.

100 mls. of de-ionised water were poured into the mixing-chamber, and 120 mls. of alcohol were poured into the reservoir. This difference in volume counteracts the effect of the difference in specific gravity between alcohol and water so that when the reservoir and mixing chamber are connected by the siphon no re-adjustment of levels occurs.

The electric motor was then switched on so that the stirring rod rotated. The siphon bulb was compressed and released so that water and alcohol were drawn into the siphon. Tap A was then closed. Tap B was opened fully and tap C was adjusted so that liquid left the apparatus at a rate of approximately 30 drops a minute. Air bubbles were then released from the part of the apparatus between the treatment chamber and the mixing chamber by applying pressure to the polythene tube.

Smith (1958) shows that when the reservoir and mixing chamber are of equal diameter, then, if two different miscible liquids are placed one in each, the solution leaving the mixing chamber shows a linear increase in concentration of that solvent which is placed in the reservoir.

When the mixing chamber contained half as much liquid as it did originally, it was assumed that it contained 50% alcohol and 50% water. This is a good stage to transfer the apparatus to room temperature (20°C).

As soon as the reservoir was empty it was recharged with approximately 20 mls. of absolute alcohol. When this had run through to the treatment chamber, 10 mls. of absolute alcohol were added to the mixing chamber, and the motor was switched off. When the mixing chamber was empty again, a further 10 mls. of absolute alcohol were added. Dehydration was then complete and the tissue ready for embedding.

Permanganate-fixed material was improved by using gradient dehydration instead of the step-by-step method but gradient dehydration gave no improvement in results when osmium tetroxide solution was used as a fixative. With this fixative the material did

not suffer noticeably if the tissue-blocks were passed through 20, 50, 75 and 95% alcohol with only 3-5 minutes in each bath, and then passed through 3 changes of absolute alcohol leaving them for 30 minutes in each.

(iv) Embedding.

(a) With methacrylate: Esters of methacrylic acid are produced commercially for the manufacture of Perspex. They are also available for embedding biological materials for electron microscopy. The monomers are colourless liquids of low viscosity which will polymerise to form clear perspex when subjected to ultra-violet irradiation or to heat. They are supplied with a hydroquinone inhibitor added to prevent spontaneous polymerisation. This can be removed by washing the methacrylate with an aqueous solution of NaOH until the NaOH ceases to be discolourised by hydroquinone. The methacrylate must then be washed of all traces of NaOH with distilled water and then dried. Full details of the procedure are given in standard text-books on electron microscopy (Kay, D., 1961, Pease, D.C. 1960).

Methacrylate monomers are completely soluble in ethanol and acetone and penetrate easily into the specimen.

The hardness of the final block formed after polymerisation can be adjusted to be the same as that of the specimen by a suitable choice of the proportions of n-butyl methacrylate and methyl methacrylate in the original monomer. Polymerised n-butyl methacrylate is quite soft compared with the hard plastic formed by the polymerisation of methyl methacrylate. A mixture of monomeric n-butyl methacrylate (nine parts) and methyl methacrylate (one part) polymerises to form a block suitable for root-tips.

Polymerisation can be brought about by adding a catalyst known commercially as "Luperco CDB" (2,4-dichlorobenzoyl peroxide with a plasticizer, dibutyl phthalate). Two per cent Luperco CDB was added to the mixture of n-butyl and methyl methacrylates. This brought about polymerisation overnight at 60°C.

Procedure for embedding in methacrylate:

After the tissue had been thoroughly dehydrated the mixture of methacrylates with added catalyst was added drop-by-drop to the final change of absolute alcohol until it contained about 10% methacrylate. The tissue was then transferred to a mixture of equal quantities of methacrylate and absolute alcohol and left for 30 minutes. Meanwhile gelatin capsules with a shallow layer (5 mm. deep) of polymerised methacrylate at the bottom were filled with methacrylate and catalyst. The tissue blocks were then transferred to capsules and orientated as desired. The full capsules were put in an oven at 60°C and left for the methacrylate to polymerise.

The setting of methacrylate is uneven and accompanied by a large shrinkage (up to 20%). Consequently the specimen may be damaged during polymerization. Although such damage can be reduced by the addition of 0.01% uranyl nitrate to the monomer (Ward, 1958) and by the use of ultra-violet irradiation instead of heat to polymerise the methacrylate, and by using partially pre-polymerised methacrylate it was considered desirable to use an

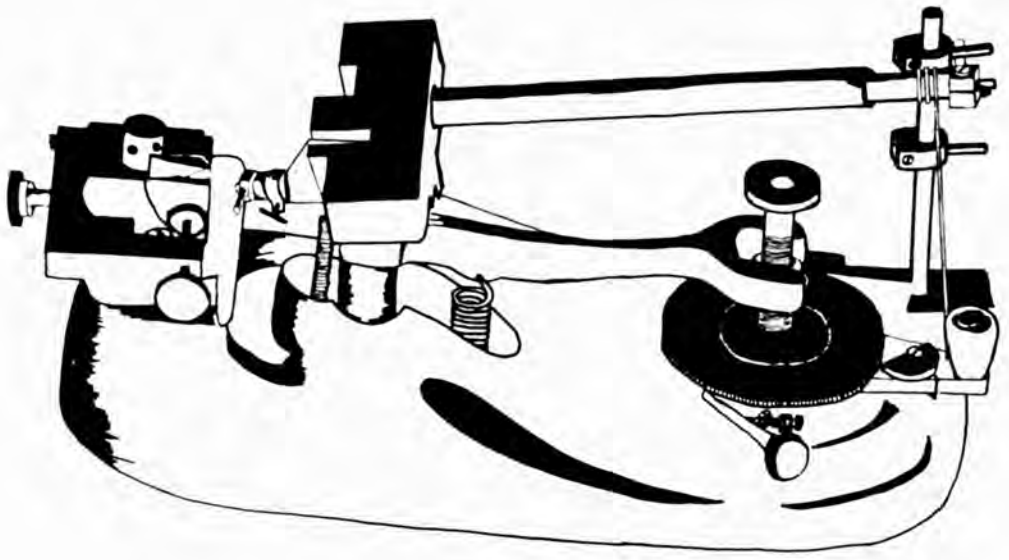
Epoxy resin as an alternative embedding medium, since they set uniformly with little shrinkage.

(b) With Epikote 812 - an Epoxy resin: Epikote 812 is the British equivalent of the American product Epon 812 and it can be obtained from the Shell Chemical Company. It was recommended for embedding biological materials for electron microscopy by Finck (1960) and Luft (1961). It has a low viscosity and penetrates the specimen far more readily than Araldite. It resembles Araldite in having a small shrinkage on curing and in maintaining its three dimensional structure under electron bombardment, and in these respects it is preferable to Methacrylate. It is preferable to Araldite, because the sections show greater contrast in the electron microscope than comparable Araldite sections and OsO_4 fixation can be used in conjunction with Epikote 812 without additional staining. Full details of the procedure for embedding in Epon 812 are given by Luft (1961) and these were followed in all details including the use of propylene oxide between dehydration and impregnation with resin. The resin was cured for 24 hours at 60°C. The component resin mixture used was three parts of "A" to two of "B" plus 1% accelerator.

(v) Microtome sectioning.

(a) The microtome: The resolution that can be obtained with the electron microscope is determined partly by the performance of the microscope and partly by the nature of the specimen. The total thickness of the specimen is often the limiting factor for the resolution. In order to obtain sections which were sufficiently thin to obtain the highest resolution possible with the microscope, a microtome built for cutting sections for the light microscope was modified so that it would hold a glass knife instead of a steel knife. A far better cutting edge can be obtained by breaking glass than by sharpening steel. Another modification involved building a mechanical advance mechanism which brings the specimen a very small distance forward towards the cutting edge between the section. It is important that the block should not pass near the knife on the return stroke, or the block and knife will be damaged. Consequently, a further modification to the original microtome was made which involved a mechanism for moving the block away to one side of the knife on the

return stroke. A diagram of the modified microtome as designed by Dr. D.C. Spanner and built by Mr. F. White, is shown in Figure 23.



(b) Trimming and mounting the specimen: Epon blocks are very hard to trim. A chisel was found to be a convenient tool for trimming a block when the material was excentric in the block, but it was easier to turn the block on a lathe if the tissue was centrally placed in the block until the diameter was approximately 3 mm. in the region of the tissue. Final trimming was done with a razor blade.

In spite of their hardness to trim Epikote blocks section well with a good glass knife.

Methacrylate blocks are easier to trim than Epikote blocks and even the preliminary trimming can be done with an Ever Ready razor blade. However, a chisel is safer. It was found most convenient to trim methacrylate blocks to a cube of sides 3 mm. approximately and then to mount the cube on a perspex rod 15 mm. long and $\frac{5}{8}$ inch diameter using a drop of chloroform (a solvent for perspex) to stick the block onto the holder. This method of mounting facilitated orientation of the specimen.

So that the original orientation of the specimen could be determined the face of the block was cut so that of the two opposite sides which were parallel to the cutting edge, one was shorter than the other, so that the sections were shaped like a trapezium.

Final trimming of the face of the block was was completed on the microtome using a glass knife. Sections 1 μ thick were cut and examined under a

phase contrast microscope to determine when the desired part of the block had been reached.

(c) Preparation of glass knives: Glass knives were prepared from strips of well annealed plate-glass approximately 1½" wide and ¼" thick.

Using a steel disc glass cutter, a series of ¼" straight scorings were made 1" apart at right angles to the long axis and alternating along the two opposite long margins of the strip.

The strip was then broken along these scorings using specially prepared V-shaped pliers. With a little practice a set of rectangular blocks 1½" x 1" x ¼" was obtained. Care was taken not to handle these blocks along the part of the breakage line which had not been scored since it was there that the knife edges were to be formed. These were made by scoring a line at approximately 45° to the future cutting edge, and applying, with slight pressure, a white-hot ⅛" pyrex glass rod to the scoring so that the block broke in two. Each of these two pieces sometimes had a good cutting edge, but more often only one was satisfactory. Examination of

the edge under a binocular microscope using reflected light revealed which knives were good and which should be discarded (Pease, D.C., 1960; Sheldon, H., 1957).

(d) Collecting and mounting the sections: As the sections were cut they floated onto a liquid (20% ethanol) surface at the knife edge. To prevent the ethanol trickling away from the knife edge a piece of "Aquaplast" (waterproof sticky plaster) 4 mm. wide was stuck on the glass knife approximately 4 mm from the cutting edge.

As the sections were cut they formed a ribbon perpendicular to the knife edge. When the ribbon was about 2 mm. long, filter paper impregnated with xylol was held about 2 cm. above it until the ribbon expanded.

A formvar coated grid was then lowered onto the ribbon so that the ribbon was picked up across the diameter of the coated side of the grid.

Formvar films were prepared from a solution of polyvinyl formaldehyde (Formvar) in chloroform as described in standard textbooks on Electron Microscopy (Kay, 1961, p.51). Films which showed silver interference colours were thin enough to use.

The grids with mounted sections on were left for at least 5 minutes to dry in a clean petri-dish lined with filter paper before they were examined in the microscope.

(vi) Staining the sections: The sections were collected on a grid in the normal way and allowed to dry. The grid was then floated on the surface of the staining solution with the sections downwards. Immediately after staining the sections were washed by holding the grid with forceps and gently flushing the grid with distilled water. The grid was then left to dry.

A saturated aqueous solution of uranyl acetate was used as a general stain. The sections were left in the stain for an hour at room temperature, in the dark to prevent photodecomposition.

B. The Electron Microscope.

Most of this work was done using one of the original Siemens pattern "Ubermikroskop" (serial number 34). It was operated with a beam voltage of 50 kilovolts and using an objective aperture of 50 micron. Although no special tests were made, it was believed that under optimum conditions a resolution of 30-40 Å was obtained. Certainly the triple nature of the nuclear membrane was resolved sometimes.

The higher resolution pictures were taken by Mr. A.D. Greenwood at Imperial College of Science and Technology using the A.E.I. EM.6.

C. Photography.

Photographs were taken using Ilford Fine Grain Safety Positive 35 mm. unperforated film at instrumental magnifications of 1,000 to 6,000 and developed with I.D.2. The film was transported on an improvised film camera. This had an advantage over the original plate camera since at least eight exposures, instead of one, could be made before reloading. Considerable trouble was experienced at first from static discharge on the film; the fact that these were registered with identical shape on two adjacent frames showed that they occurred as the

film was being wound onto the take-up spool. It was overcome by arranging for the film to be transported on an endless strip of nylon. This limited the length which could be accommodated, but by dispensing with the coiling of the film on spools it greatly improved the ease of out-gasing, and it proved unnecessary with this arrangement to treat the films in a vacuum desiccator.

Prints were made on Kodak Bromesko WSG single weight paper.

D. Calibration of the electron microscope.

A Formvar replica of a diffraction grating supplied by the National Physical Laboratory was made for this purpose. It was important to use a replica technique which did not involve soaking the diffraction grating in water, since this would damage it. The following method was satisfactory.

The grating was coated by dipping it into a 0.5-1.0% Formvar solution in chloroform, and allowing it to drain in the vapour. To facilitate the removal of the replica it was backed with a thick film of nitrocellulose from a 2% solution in amyl acetate. The film was dried for about 15 minutes in gentle heat.

The Formvar-nitrocellulose film was removed by scoring the join to the glass support with a razor blade. The film was then breathed on heavily. This made it possible to run the corner of a razor blade under the composite film along one side. The whole film could then be detached gently with tweezers.

The Formvar-nitrocellulose film was then floated on water and grids were coated with it as described for coating grids with formvar films (Kay, 1961, p.51).

The nitrocellulose backing was removed by dissolving it in amyl acetate.

Photographs of the Formvar replica of the diffraction grafting were taken at each of the possible magnifications.

E.(i)Evaluation of fixation and embedding of material.

During polymerisation methacrylate shrinks by as much as 20%, and since polymerisation occurs unevenly throughout the block, the specimen is liable to strains and stresses during embedding. These strains and stresses result in polymerisation damage. Signs of such damage were apparent in some of the methacrylate-embedded material. For example the cell

walls were bent back on themselves. This was believed to be an artefact caused by polymerisation, since it was never observed in blocks embedded in Epikote 812, and it was of infrequent occurrence when partially pre-polymerised methacrylate was used. Another shrinkage artefact, which may have been due to polymerisation damage, or to differential shrinkage during fixation, was the presence of empty spaces instead of granular hyaloplasm in the regions between the membranes. This occurred most frequently near the bent cell walls. Material showing either feature was discarded.

Gaps between the cell wall and the ectoplast were regarded as indicating that the fixative was hypotonic. This was the case when osmium tetroxide was used without sucrose and it was remedied by the addition of 0.015 g. sucrose per ml. of fixative.

The mitochondria were useful indicators of swelling or shrinkage. If there was no shrinkage the microvilli were straight and arranged radially. If shrinkage had occurred they were bent in various directions. If swelling had occurred the matrix between the microvilli often appeared empty in parts instead of being uniformly granular.

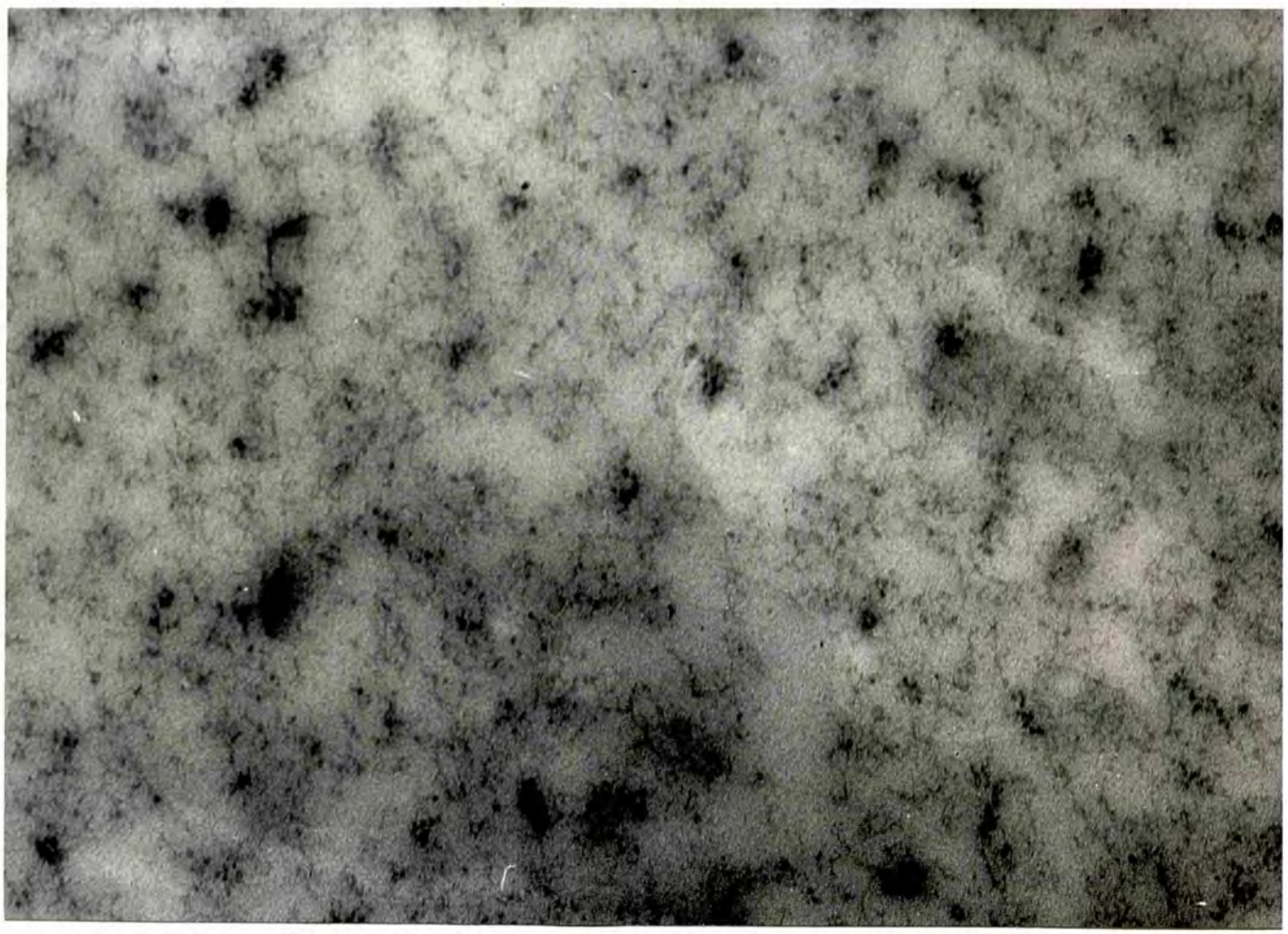
The electron micrographs of other workers were valuable as a basis for assessing the standard of fixation attained. Of special use in evaluation and interpretation of permanganate-fixed material was the paper of Mollenhauer, Whaley and Leech (1960), in which the cell ultrastructure responses to mechanical injury are discussed.

Published electron micrographs of root-cap cells are few - the majority of people discard the root-cap and concentrate on the meristem. However, Mollenhauer, Whaley and Leech (1961) have published a paper in which they discuss the function of the Golgi Apparatus in outer root-cap cells. In this paper electron micrographs of root-cap cells of Zea mais fixed in potassium permanganate are shown. In another paper by Mollenhauer the permanganate fixation of central root-cap cells is discussed and the electron micrographs are comparable with the ones included in this thesis. (Mollenhauer 1959)

No published electron micrographs of osmium-fixed root-cap cells could be found. This was unfortunate, because osmic acid fixation followed by uranyl acetate staining showed very few ribosomes in the root-cap cells, and it would have been useful

to discover whether other workers have found the same paucity of ribosomes.

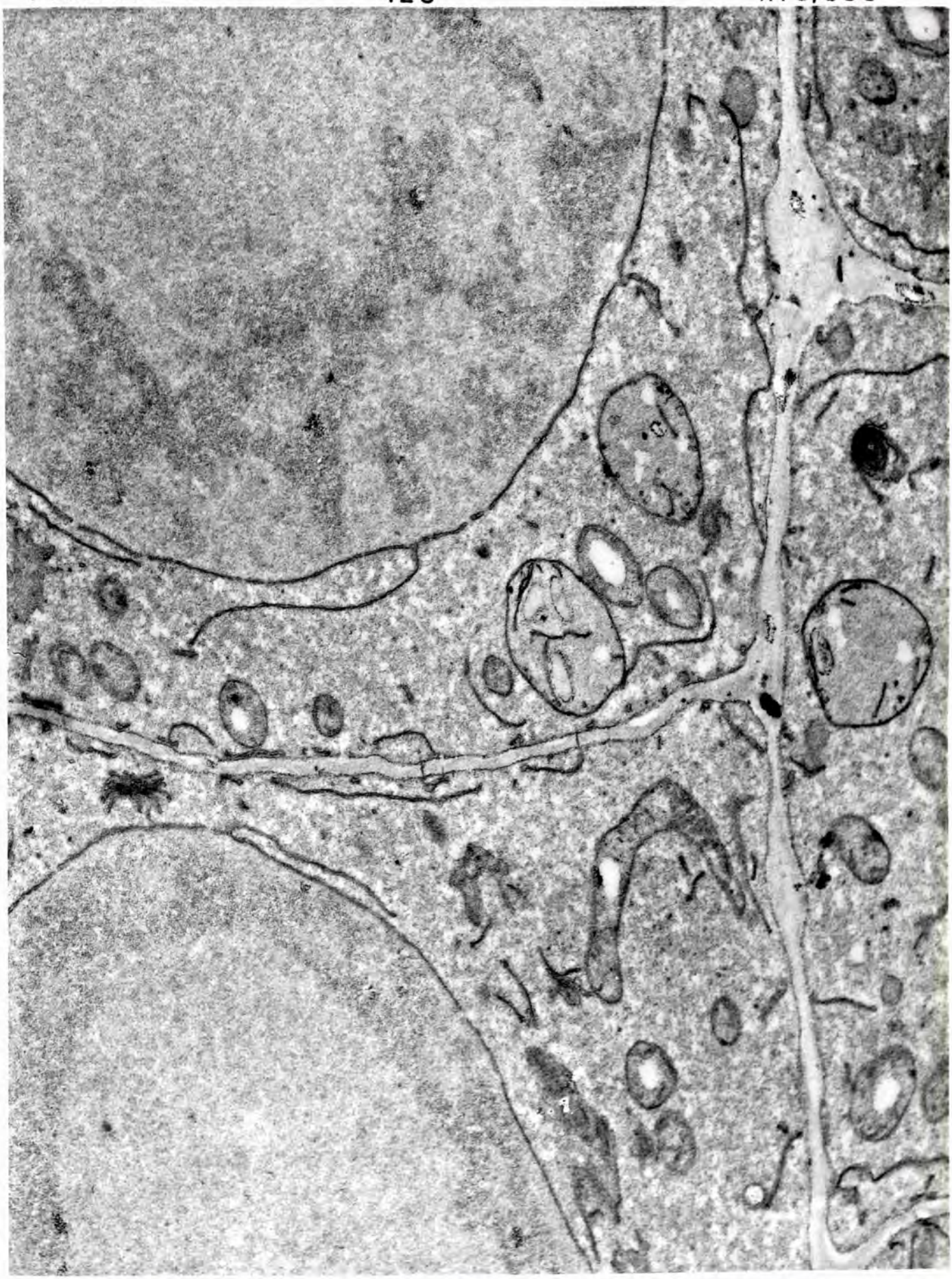
FIGURE 24. RIBOSOMES IN THE ROOT-CAP. X 55,000. FIXATION AFTER CAULFIELD. STAINED WITH URANYL ACETATE.



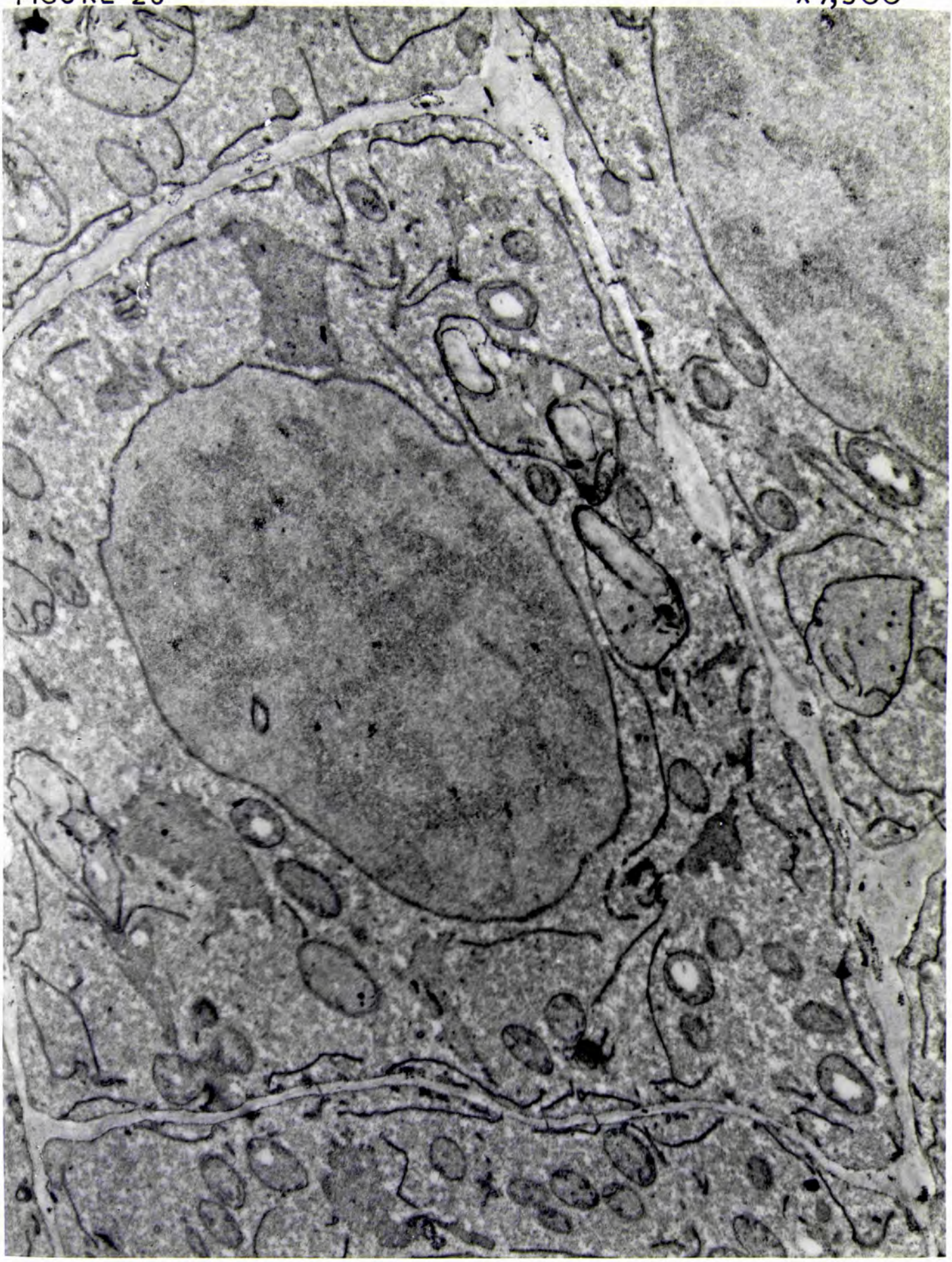
It is possible that extraction of RNA or protein from the microsomes had occurred during dehydration. Or it may be true that root-cap cells do contain relatively few ribosomes. One indication that this may be the case was found by comparison of root-cap cells which had been dehydrated by the rapid step-by-step method with those which had been through gradient dehydration. With both treatments there

were few ribosomes. If the paucity was due to extraction one might expect to have greater extraction by the gradient method than by the rapid step-by-step method, and hence fewer ribosomes. Comparison of the density of ribosomes in the meristematic cells with that in the root-cap cells of the same root showed a considerably higher ribosome concentration in meristematic cells. This indicates that extraction of ribosome material is probably not occurring to any great extent. It seems unlikely that extraction would be specific for any one type of root cell.

Because published photographs of root-cap cells are scarce it was decided that the meristem cells should also be studied and comparisons made between the fixation of these and of those in published papers. In all cases the appearance was similar and such similarity was taken as an indication that fixation was reasonable (Figures 25 and 26), and it was assumed that this being so in root meristem cells, it was probably also true for root-cap cells.



Meristematic cells from the root of Vicia faba fixed with $KMnO_4$ and embedded in methacrylate. Taken with EM.6.



Meristematic cells fixed with $KMnO_4$ and embedded in methacrylate.

Taken with EM.6.

(ii) Speed of penetration of the fixative. An important characteristic of the fixative used for this particular work was the speed of penetration, because it was required that movement of cell organelles should not occur except as a result of the action of gravity during the experiment prior to fixation.

In order to discover whether detectable movement of organelles occurred during fixation, roots which had been horizontal for sufficiently long for the starch grains to have dropped to one side of the cell (20 minutes) were put in 2% $KMnO_4$, maintaining the experimental position until they were immersed in the fixative, then they were immediately turned about their long axis through 180° and left for 30 minutes. After this time they were embedded and sectioned in the plane which was vertical during the experiment. It was found that detectable movement of starch grains had not occurred during fixation.

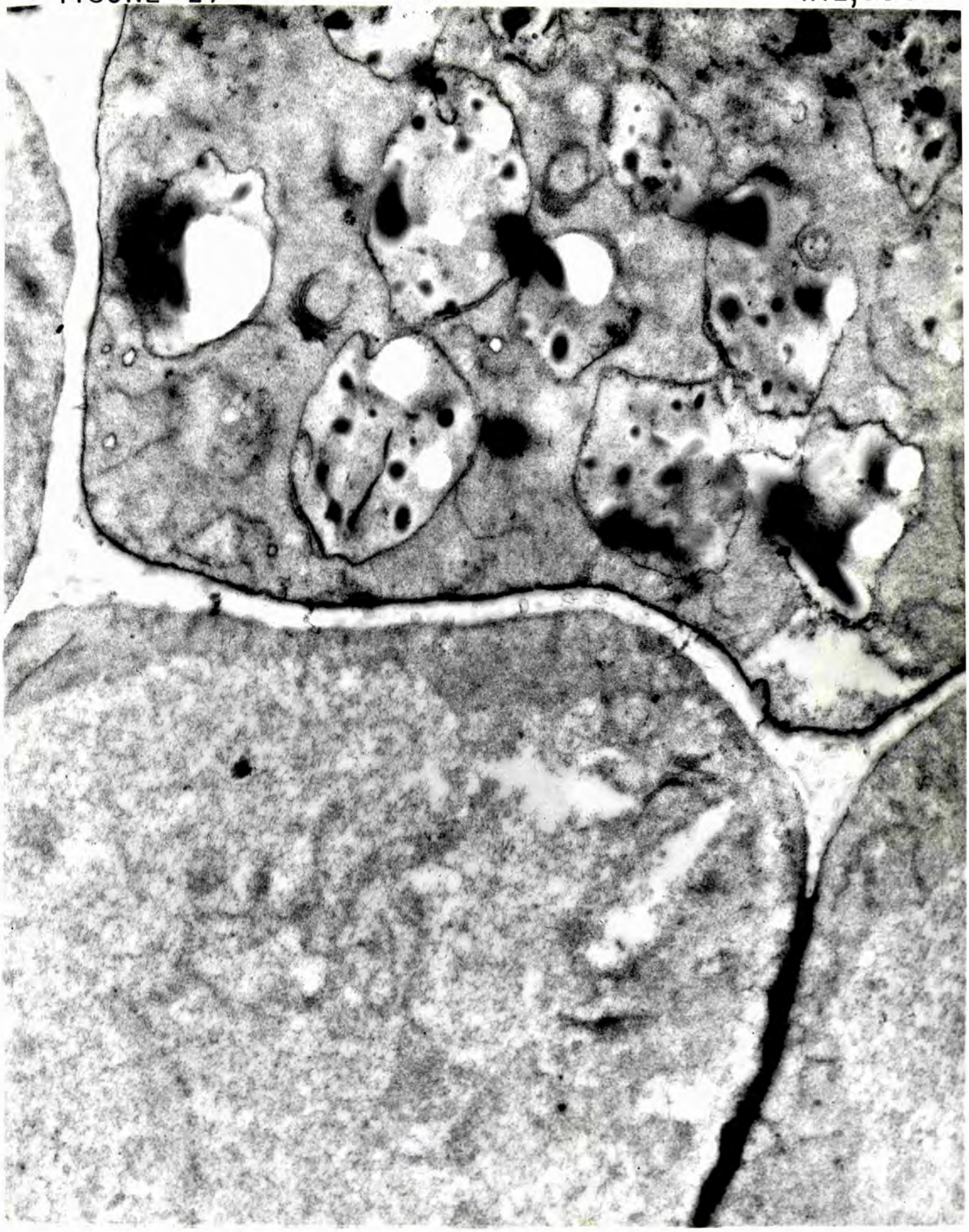
A further experiment was made in which roots which had been growing normally were fixed upside-down. In this experiment normal distribution of starch was observed. In both experiments mitochondria appeared to be randomly distributed throughout the

cytoplasm. This was also the case when the experimental position was maintained during fixation, indicating that obvious unilateral distribution of mitochondria does not occur during horizontal stimulation (a statistical analysis of this is presented later in the thesis), and that redistribution of mitochondria does not occur during fixation.

(iii) Fixation artefact caused by unilateral penetration of fixative. The only consistent staining difference between stimulated and unstimulated root-cap cells occurred at the boundary between the cell wall and the cytoplasm. It was observed that an electron dense layer occurred along this boundary, only at the physically lower sides of the root-cap cells, whether they were fixed with $KMnO_4$ or OsO_4.

A considerable amount of time was spent in trying to elucidate the nature of this dark layer.

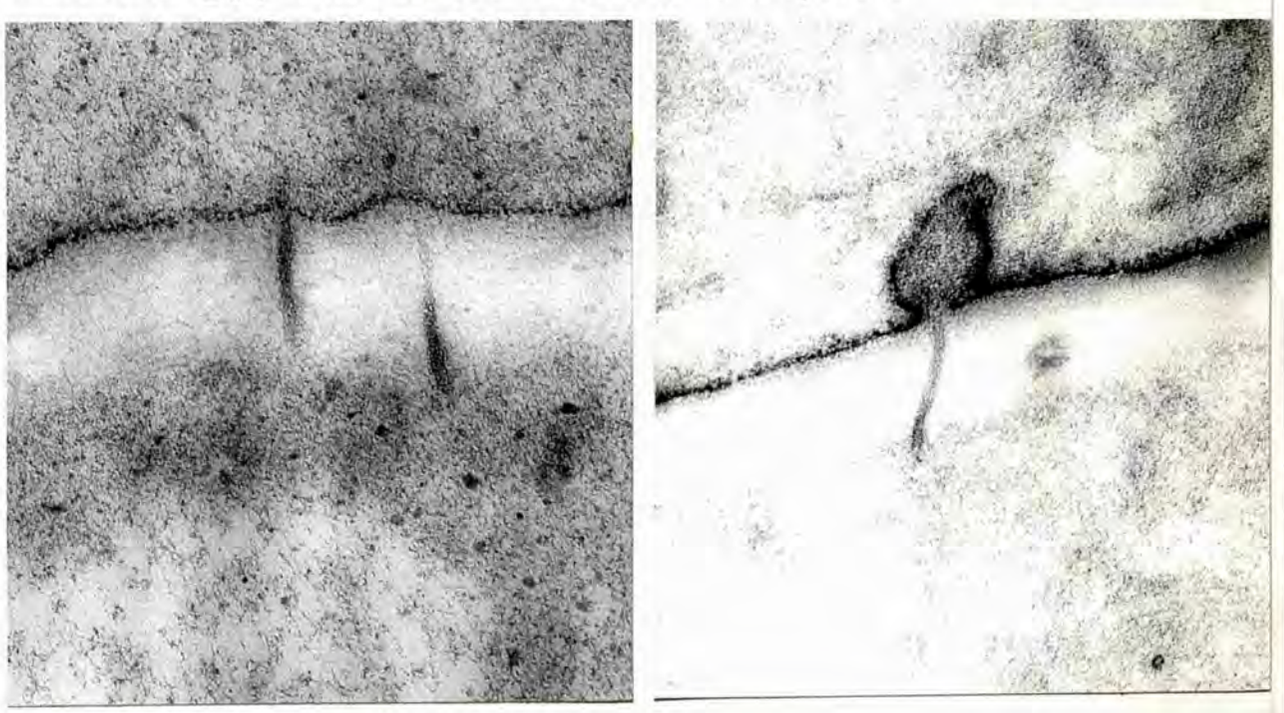
It was observed in unstimulated cells at the morphologically (and physically) lower ends of the root-cap cells. After 20 minutes horizontal stimulation it still occurred at the morphologically lower ends of the cells, but it also occurred along the physically lowermost side-walls of the cells.



Root-cap cells showing dark layer at the cell-wall/cytoplasm boundary underneath the plastids. Fixed with $KMnO_4$. Taken with EM.6.

A large number of cells was examined and the dark layer was found to be consistent in its position and in its occurrence. It was thicker and denser in cells which were regarded as being over-fixed and in which the plasma-membrane was not preserved, but it also occurred in cells which were reasonably well-fixed. High resolution pictures were taken. In some places the layer was represented as a granular layer of various sized particles and of varying thickness. In a few places it appeared more like a darkly staining single or double membrane. But in no cases was the fixation of the other cell components sufficiently good to conclude anything definite about the size and nature of the particles which comprised the dark layer.

FIGURE 28: THE DARK LAYER X 50,000



It was difficult to imagine such small objects as these particles appeared to be, falling so very precisely and compactly into such a position after only 20 minutes horizontal stimulation. The only plausible explanations of it seemed to be that either

1. the material was very heavy. But if that is so, why did the particles remain along the morphologically end wall when the root was horizontal?

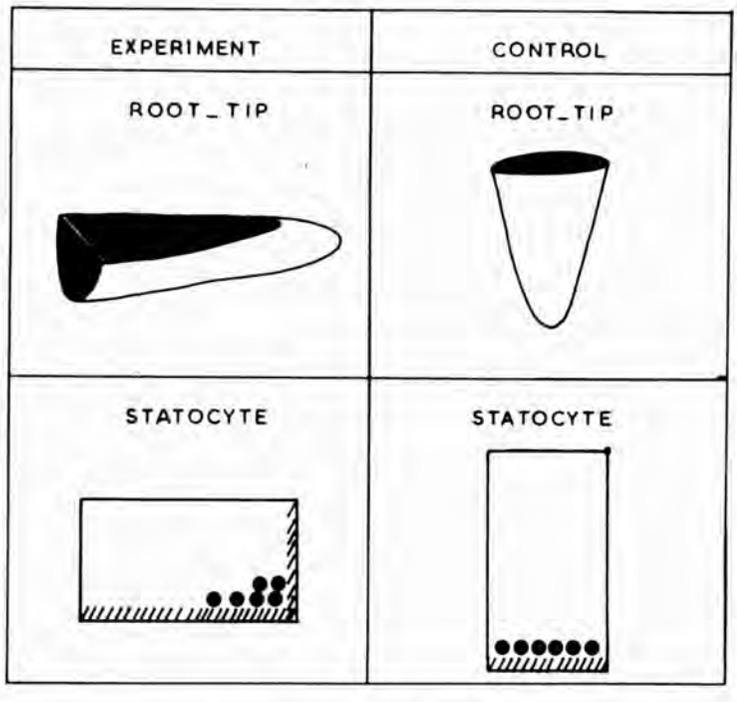
or 2. that it was attracted by other forces to this position

or 3. it was material produced by a changed environment in that region

or 4. it was a product of fixation resulting from a changed chemical environment close to the wall.

The size of the particles was the same as the size of ribosomes, but unlike ribosomes the particles were clearly seen in $KMnO_4$-fixed material. Uranyl acetate staining did not intensify them at all.

Figure 29: This illustrates the position of the cuts made in the horizontally stimulated roots and the vertical controls. It also shows the position of the dark layer in each.



The possibility of artefact was realised when the tangential cut made for orientating the specimen after it had been horizontal was considered. No tangential cut was made in the control roots and this was an obvious mistake in the experimental method. Perhaps unilateral penetration of fixative occurred from the cut surfaces and caused the formation of a dark layer along the walls furthest from the direction of penetration as shown diagrammatically in Figure 29.

This would explain why the dark line consistently appeared at the same side of the root-cap cells as the plastids.

To discover whether the dark layer was an artefact, the following experiment was designed (see over).

In all three experiments the result expected on the basis that the dark layer is a fixation artefact caused by unilateral penetration of the fixative, was obtained.

Figure 30: This illustrates the experiment designed to show whether the dark line is an artefact depending on the direction of penetration of the fixative.

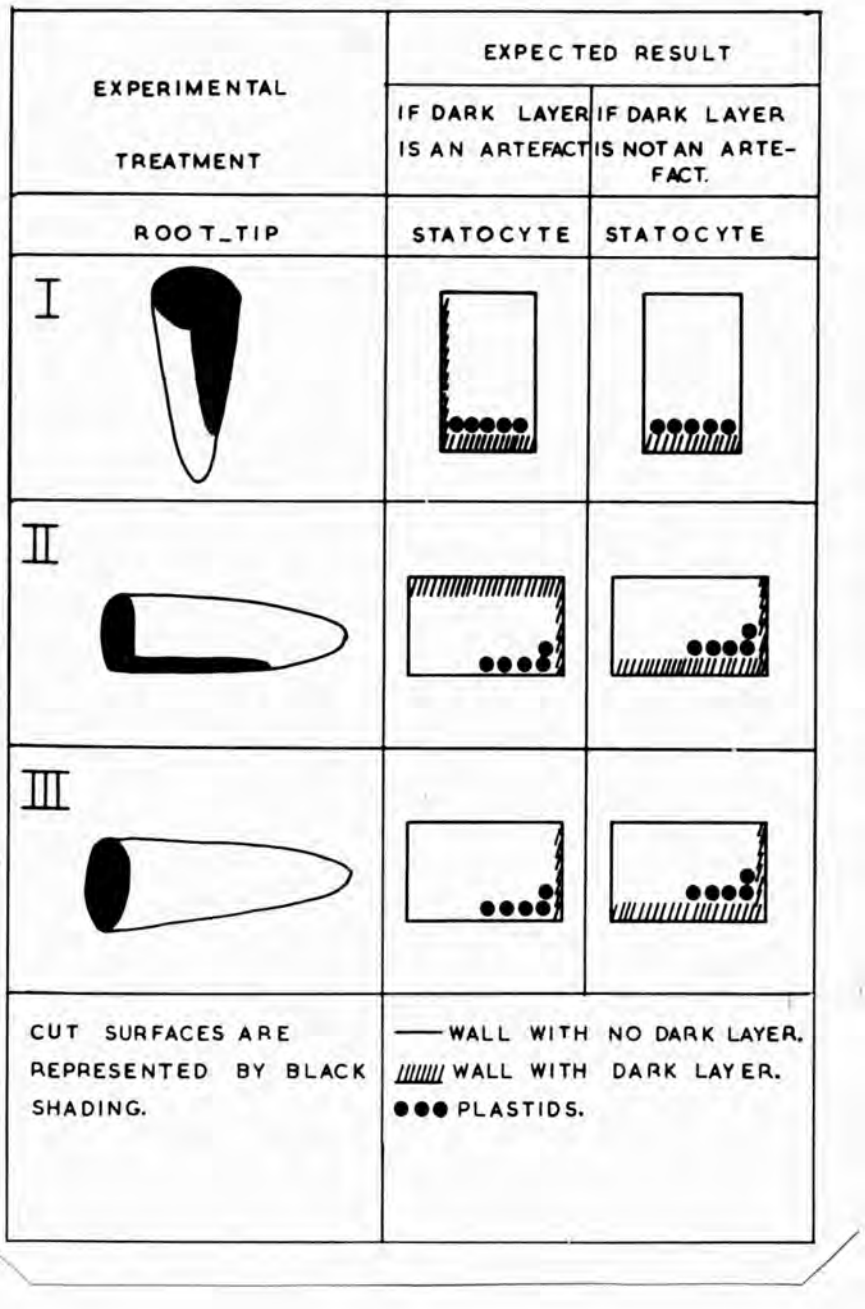
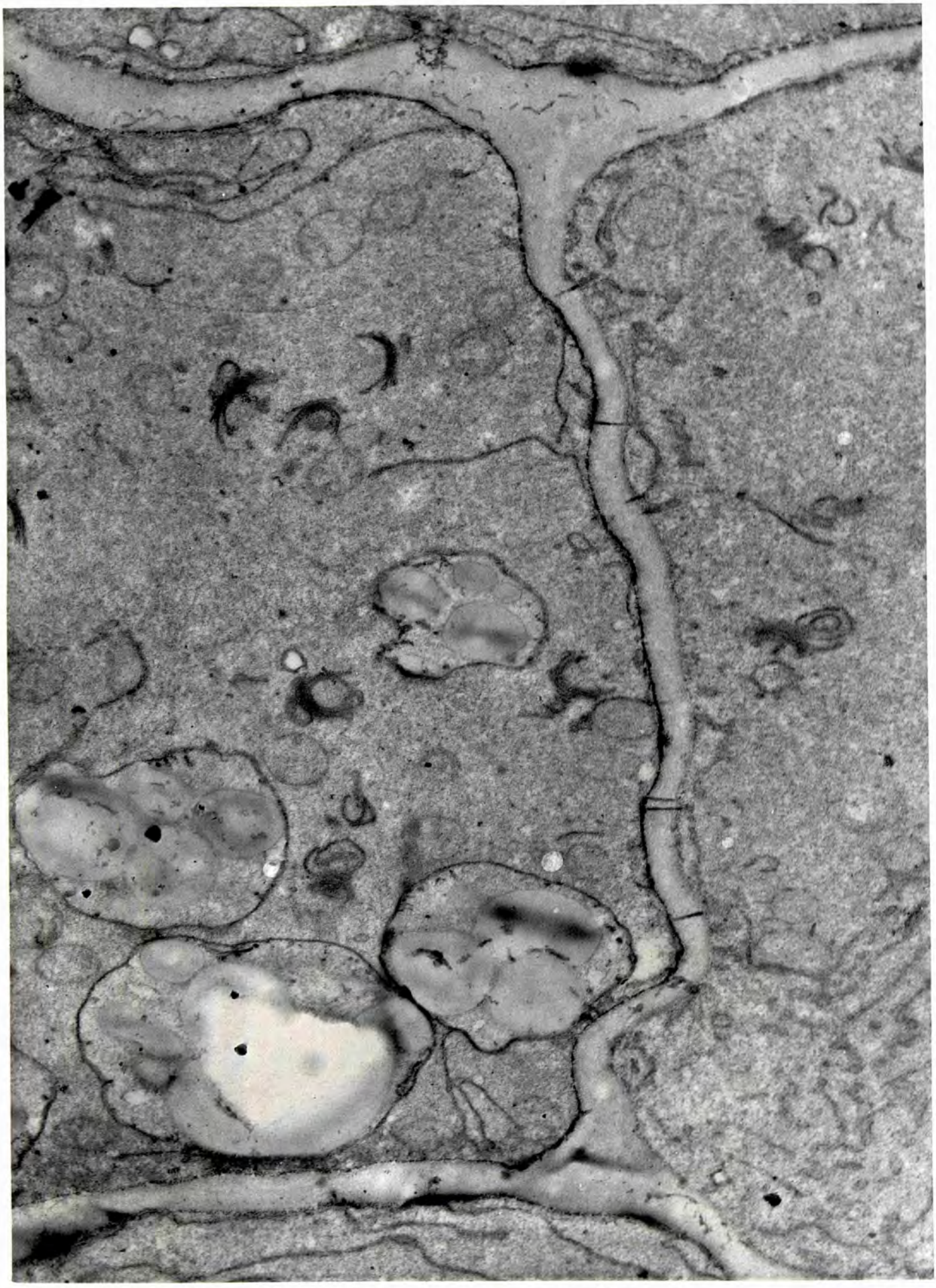


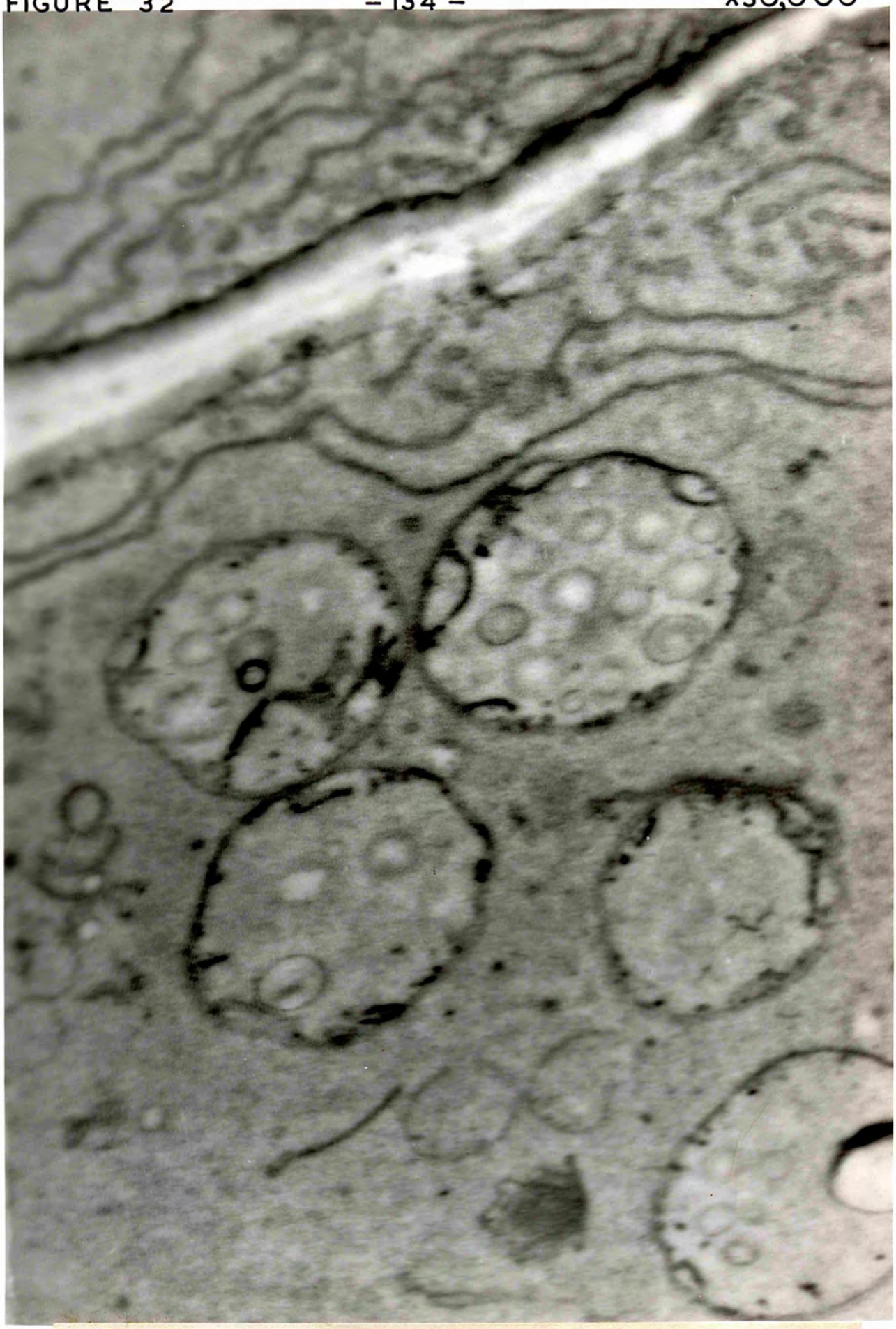
Figure 31: Cap cells from a root which had been horizontal for 20 minutes. Note that the dark layer has formed at the boundary of the cytoplasm with the cell wall opposite the plastids. The tangential cut was made at the lowermost side of the root after stimulation.

$KMnO_4$. Methacrylate. EM.6.

FIGURE 31

X16,000





The dark layer has formed on the side of the wall opposite to the plastids. This is the side of the root at which tangential cut was made.
$KMnO_4$. Methacrylate. Siemens.

(iv) Conclusions regarding fixation and embedding techniques. Providing that the generally accepted pattern for the ultrastructure of the cell is a true representation, the two fixatives (Caulfields buffered osmium tetroxide with added sucrose, and 2% unbuffered potassium permanganate) gave reasonable fixation.

Penetration of $KMnO_4$ into the tissue was sufficiently rapid to prevent detectable movement of organells during fixation. Osmic acid did not penetrate so quickly.

There was considerably less polymerisation damage with Epikote 812 than with methacrylate. Consequently the fine structure of the cells and the intracellular relationships were better preserved when Epikote 812 was used. Methacrylate embedding was improved by the use of partially pre-polymerised methacrylate.

Epikote sections seemed to suffer less degradation during irradiation by the electron beam than those cut from methacrylate blocks.

F. The Ultrastructure of the root-cap cells.

The chief components of the root-cap cells as they are seen after permanganate and osmic acid fixation are illustrated in the photographs of Figures 33, 34, 35 and 36.

Figure 33: Root-cap cells. $KMnO_4$ x 10,000. Siemens.

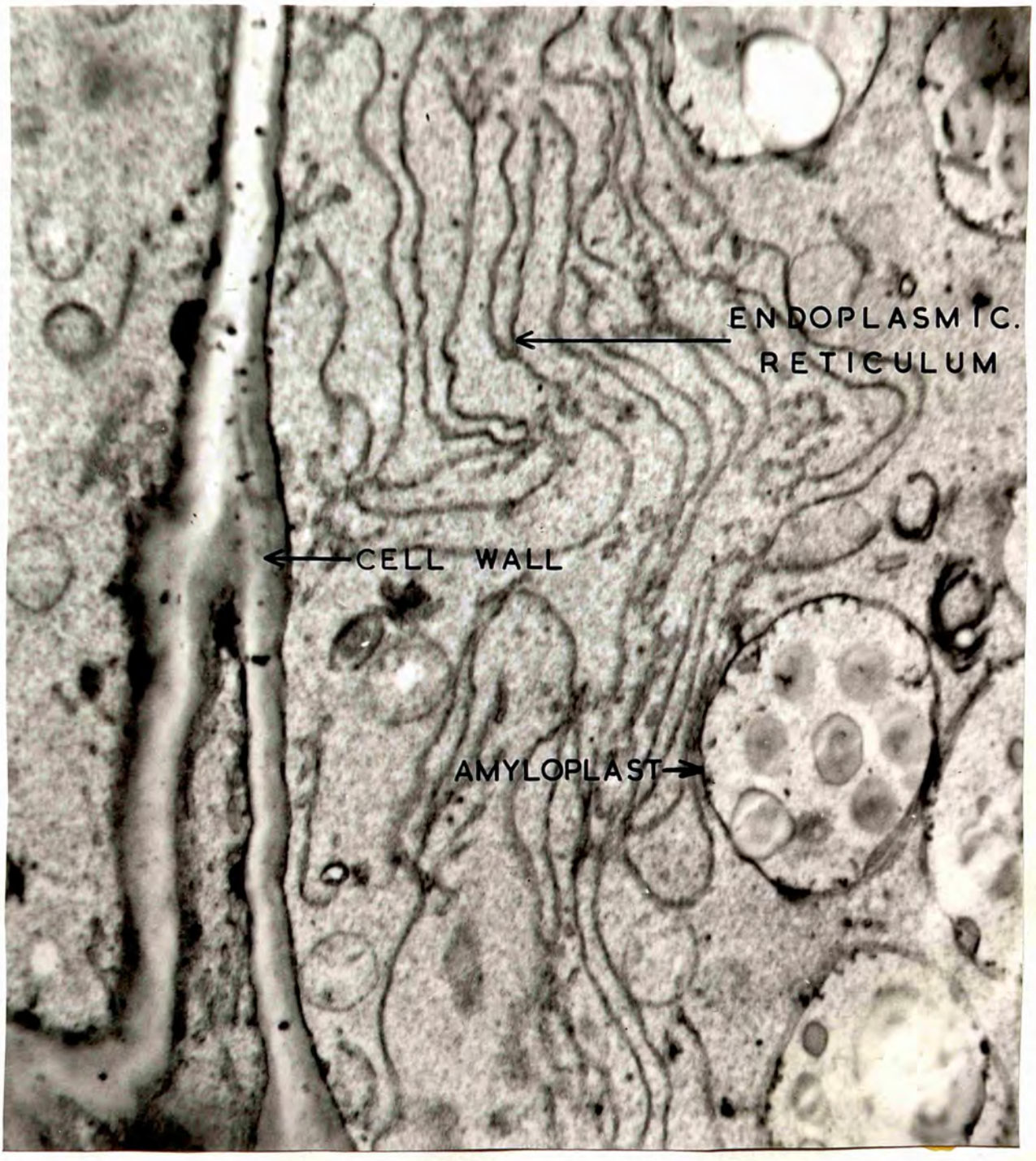
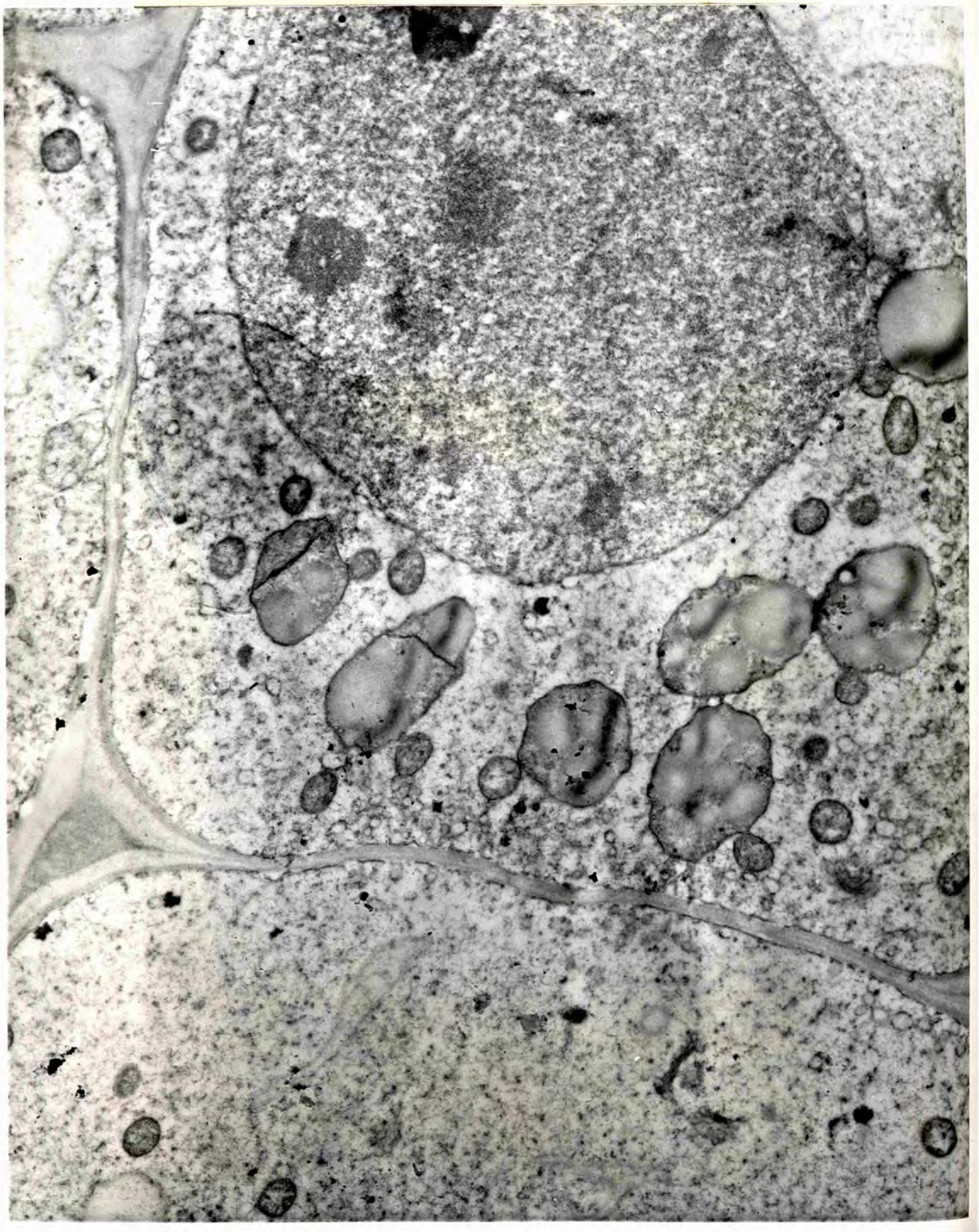
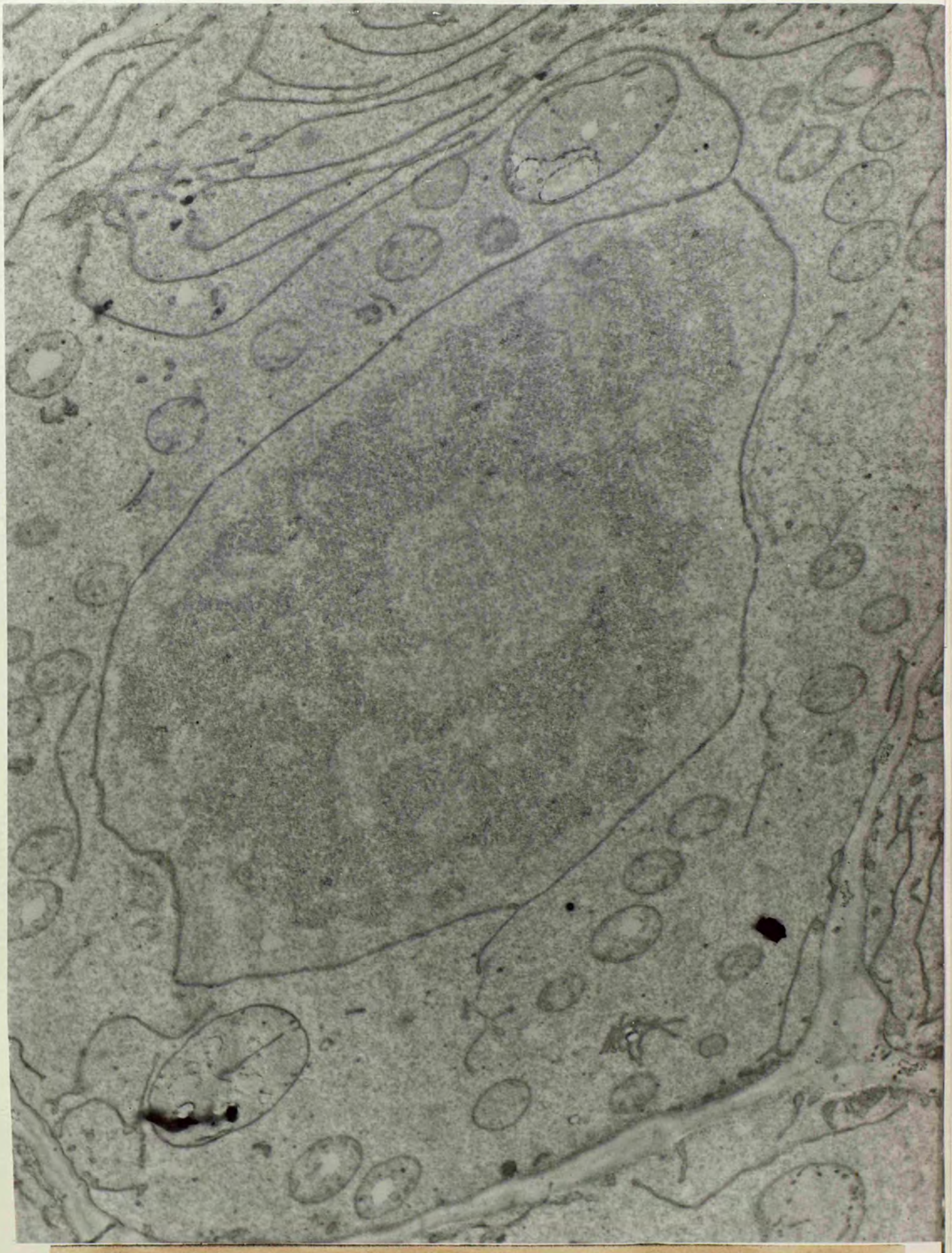


Figure 34: Root-cap cell fixed with Osmic acid.

FIGURE 34 - 137 - X12,000

Root-cap cell fixed with Osmic acid. EM.6.





Root-cap cell fixed with $KMnO_4$. Note continuation of endoplasmic reticulum and nuclear membrane. EM.6.

Figure 36a: x 55,000. OsO_4. EM.6.

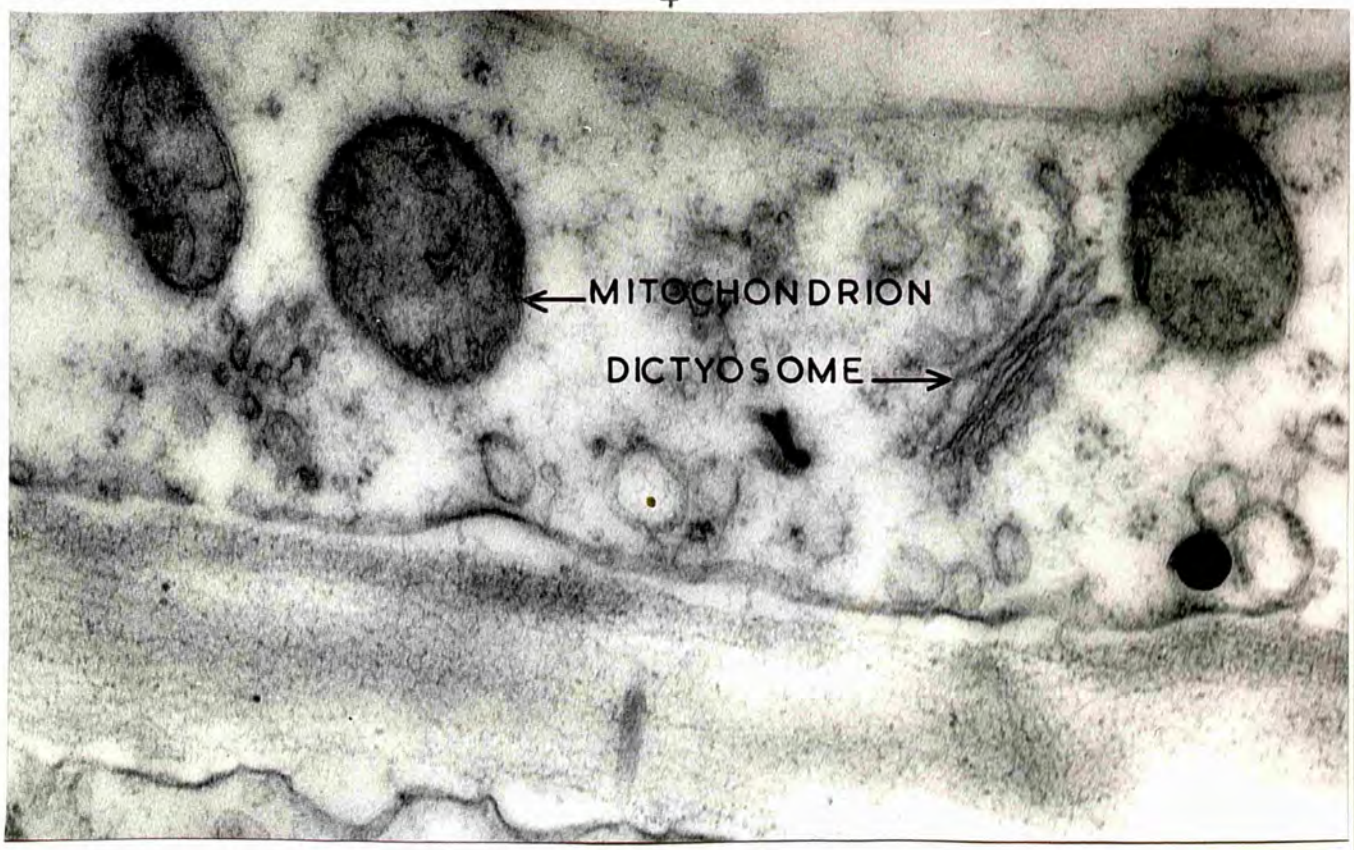
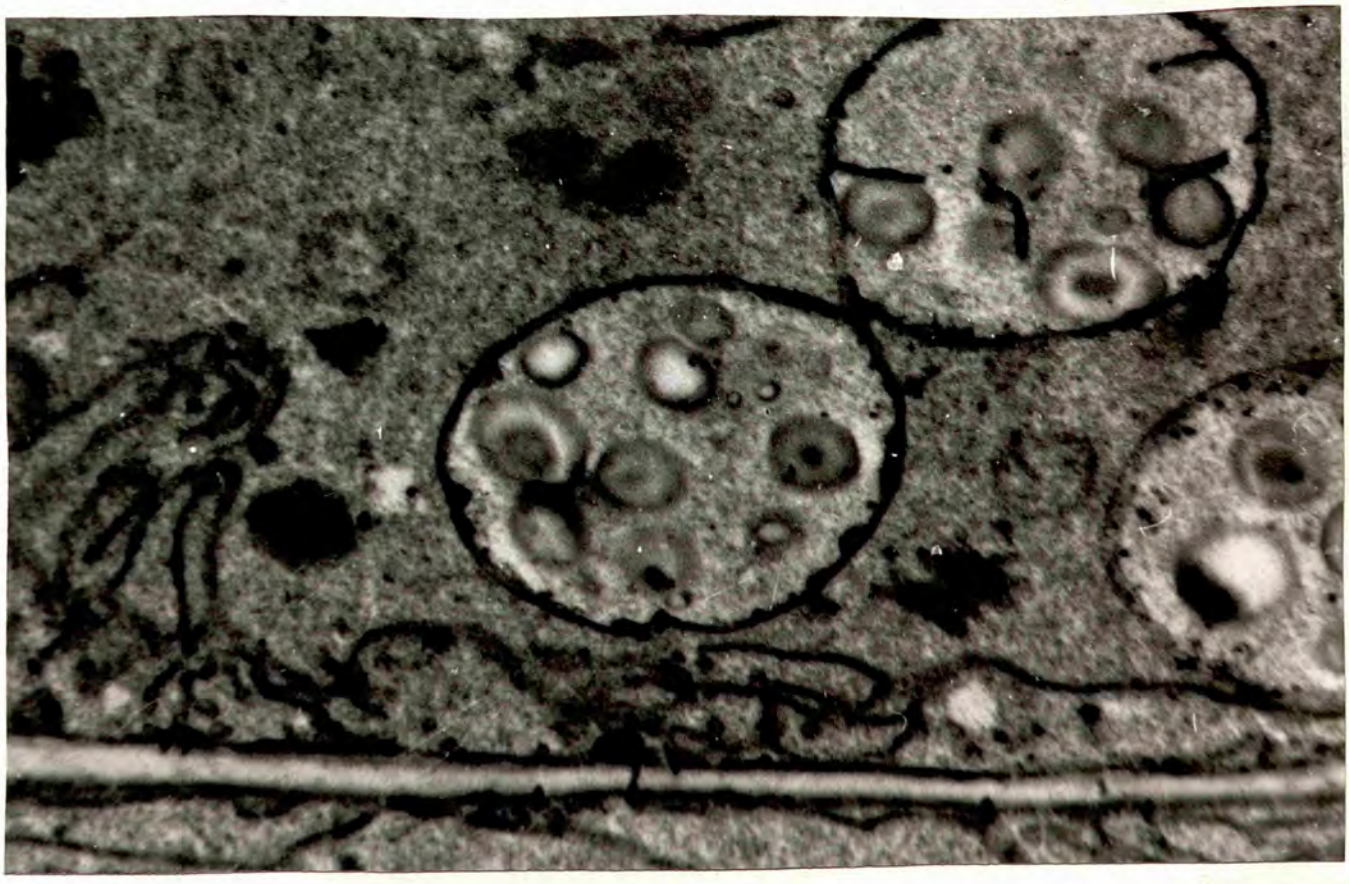


Figure 36b: x 15,000. $KMnO_4$. Siemens.

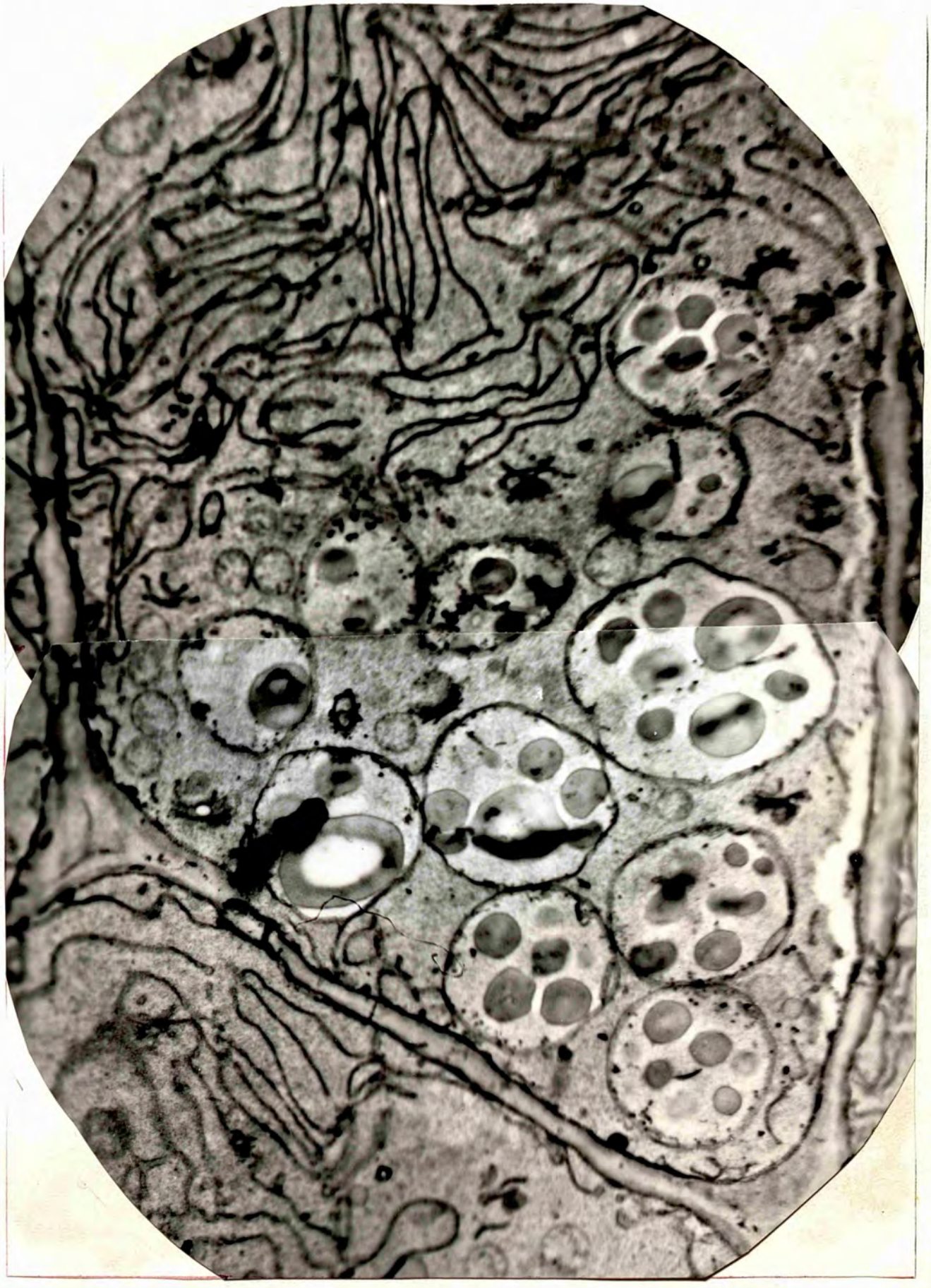


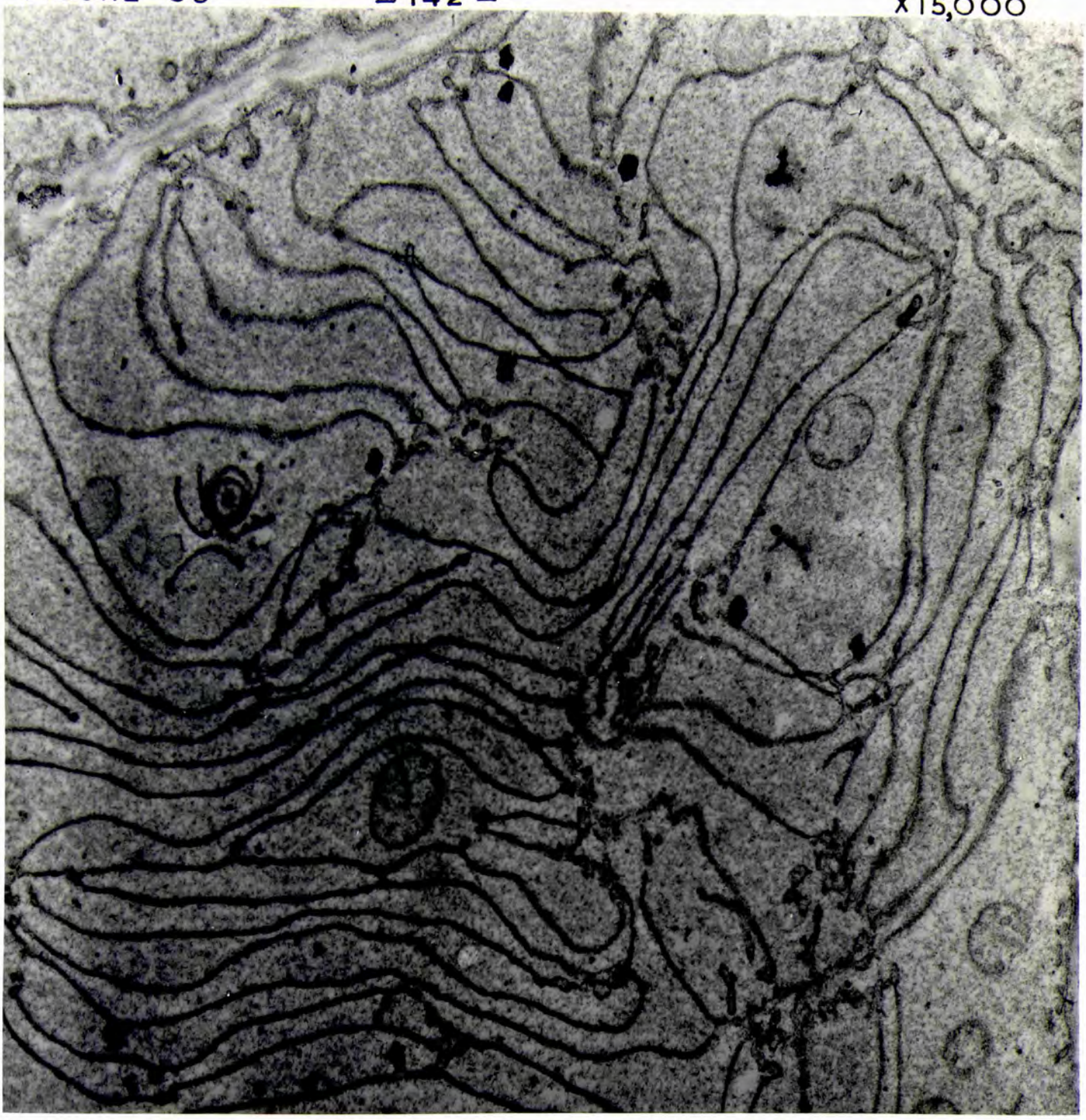
The ground substance differed in appearance according to the fixative. If $KMnO_4$ was used and the sections examined at 10 Å resolutions, the matrix appeared finely and uniformly granular. Using lower resolution (40-50 Å) the matrix appeared homogenous. When osmium tetroxide was the fixative the ground substance was seen to contain some ribosomes and was more coarsely and irregularly granular. The ribosomes were heavily stained with uranyl acetate.

The endoplasmic reticulum was quite extensive. It was sometimes seen to be continuous with the nuclear membrane (see Figure 34) and occasionally continuity of the endoplasmic reticulum was seen between adjacent cells through the plasmadesmata.

The endoplasmic reticulum was sometimes highly concentrated at the end of the cell distal from the root-tip (Figure 37). At other times it was distributed fairly uniformly throughout the available cytoplasm. Frequently the more peripheral membranes were more or less parallel to each other and to the cell wall, (Figure 33, Whaley, Mollenhauer and Leech, 1959). This pattern of endoplasmic reticulum has also been observed by B.E. Juniper (private communication) in root-cap cells of Zea mais; frequently in these cells,

Figure 37: Unstimulated cell with endoplasmic reticulum at proximal end and plastids at distal end. x 7,500 Siemens





Endoplasmic reticulum, dictyosomes and mitochondria in a root-cap cell. Fixed with $KMnO_4$ and embedded in methacrylate.

EM.6

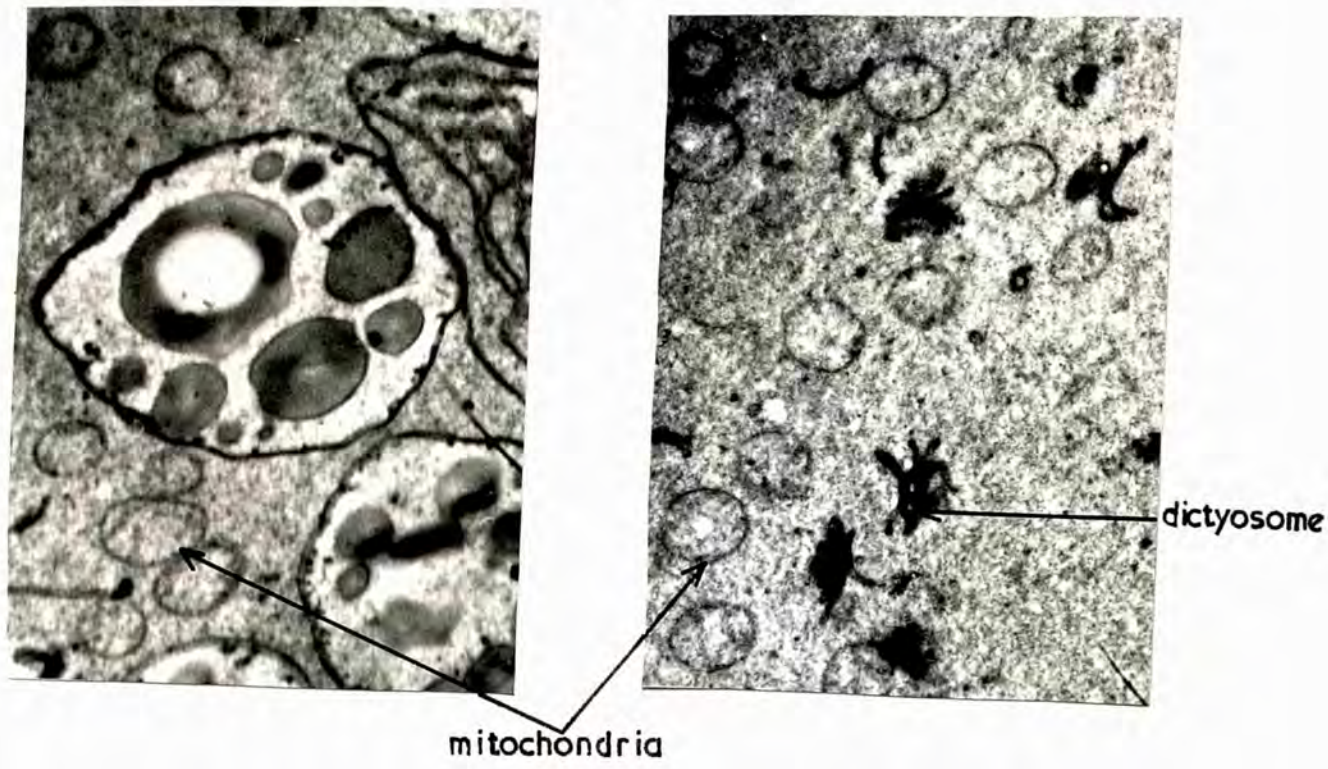
as in the cells of the root-cap of Vicia faba, "the endoplasmic reticulum is concentrated to a certain extent at the proximal end of the cell. This is not absolute, but it is undoubtedly significant", (B.E. Juniper, 1963). One profile of endoplasmic reticulum is always seen to follow back from the region of concentration to the distal end of the cell.

There were numerous mitochondria in every root-cap cell. The majority of them were circular or ellipsoid in section, with microvilli which extended radially from the periphery of the mitochondria inwards towards the centre. Usually they were less than half the length of the radius, so they were seldom continuous across the mitochondria.

The size of the mitochondria in section was variable, the range being from 0.3 - 0.8 μ diameter. This difference was due partly to differences in the plane of the sections; it was also due to variations in fixation technique. The mean size of $KMnO_4$-fixed mitochondria was 30% higher than the equivalent value for OsO_4-fixed material. Whether this was due to shrinkage during OsO_4 fixation or epikote embedding, or to swelling during $KMnO_4$-fixation was not determined.

Inherent differences in size probably contributed in part to the differences observed.

Figure 39: X 12,000. $KMnO_4$. Siemens microscope.



The ground substance of the mitochondria was usually similar in electron density and granularity to the cytoplasmic ground substance when $KMnO_4$ was used as a fixative. When OsO_4 was used, the mitochondrial matrix was more densely and finely granular than the cytoplasm of the same material.

Dictyosomes were also numerous. There were usually six or less cisternae in each dictyosome. The cisternae were up to 1 in diameter and the stack was up to 1 high. They are illustrated in Figure 34.

The distribution of mitochondria and dictyosomes appeared to be random throughout the cytoplasm. An analysis of this is presented later (page 163).

The membrane-bound amyloplasts contain a variable number of starch grains, which stained variously with $KMnO_4$; sometimes they were very electron dense (Figure 40a), while at others they were almost transparent to electrons.

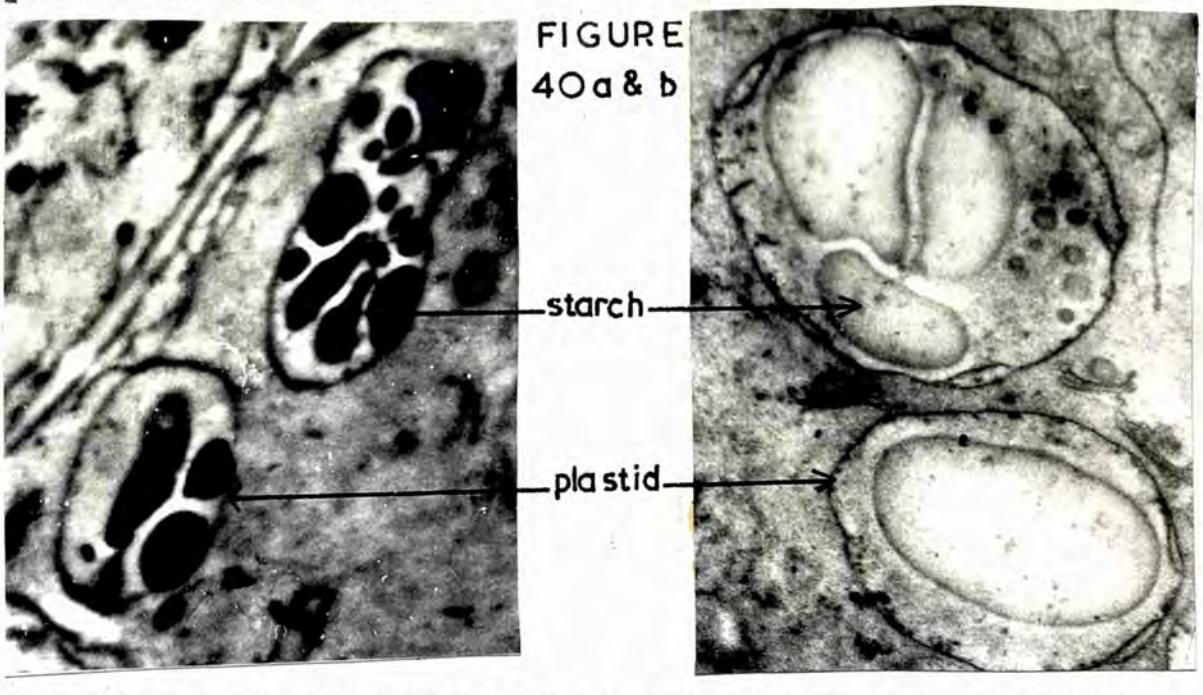
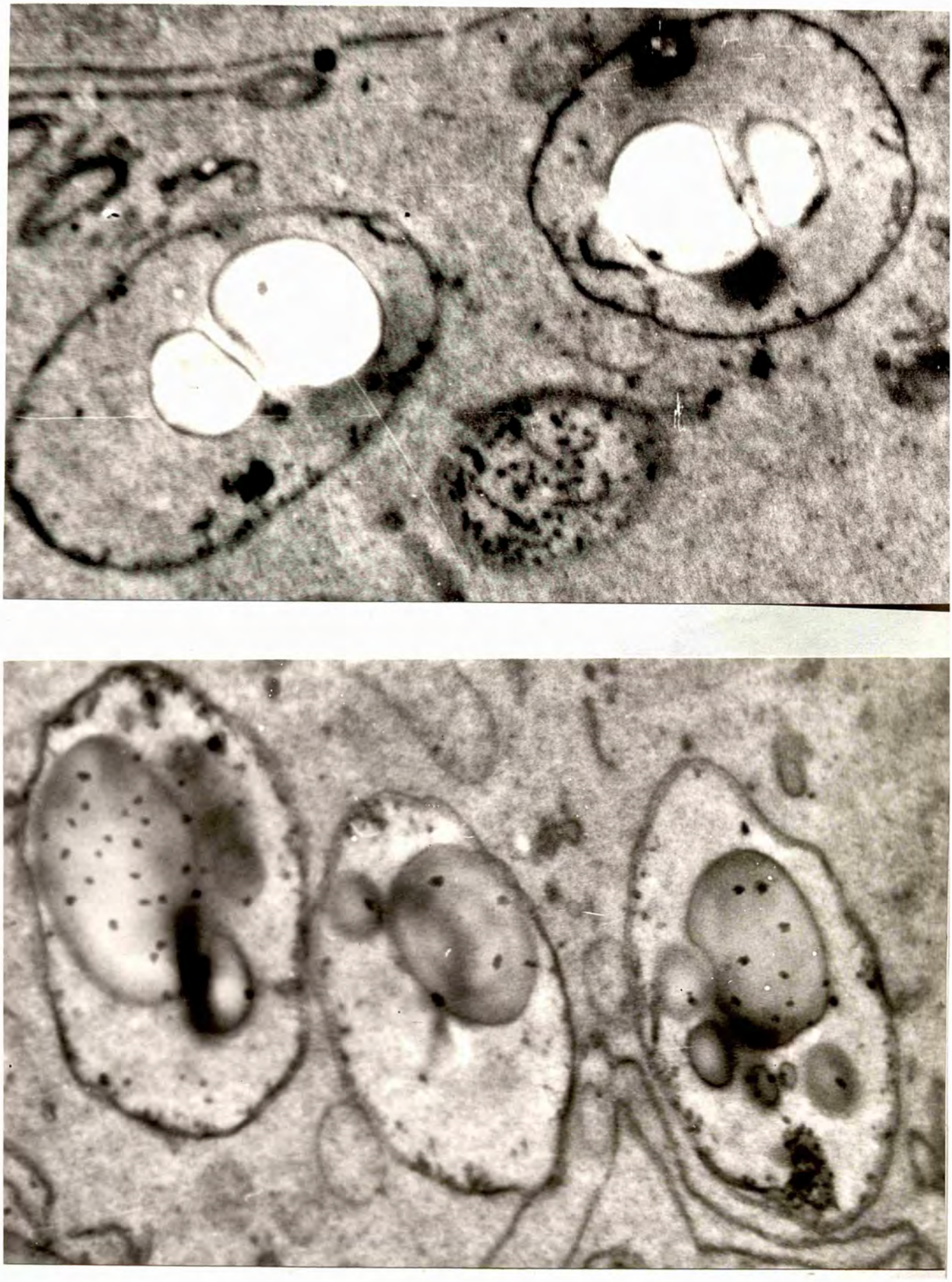
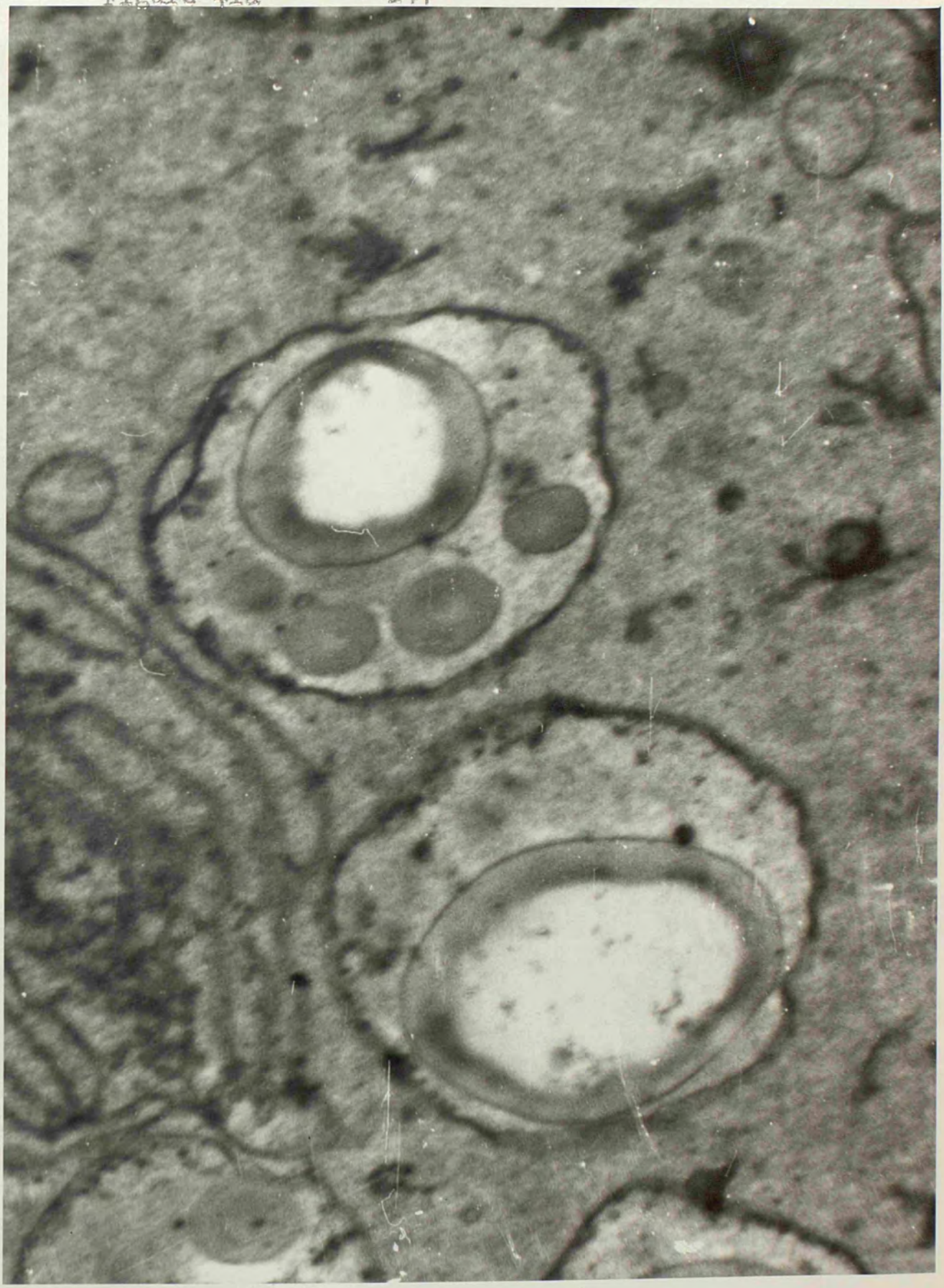


FIGURE 40a & b

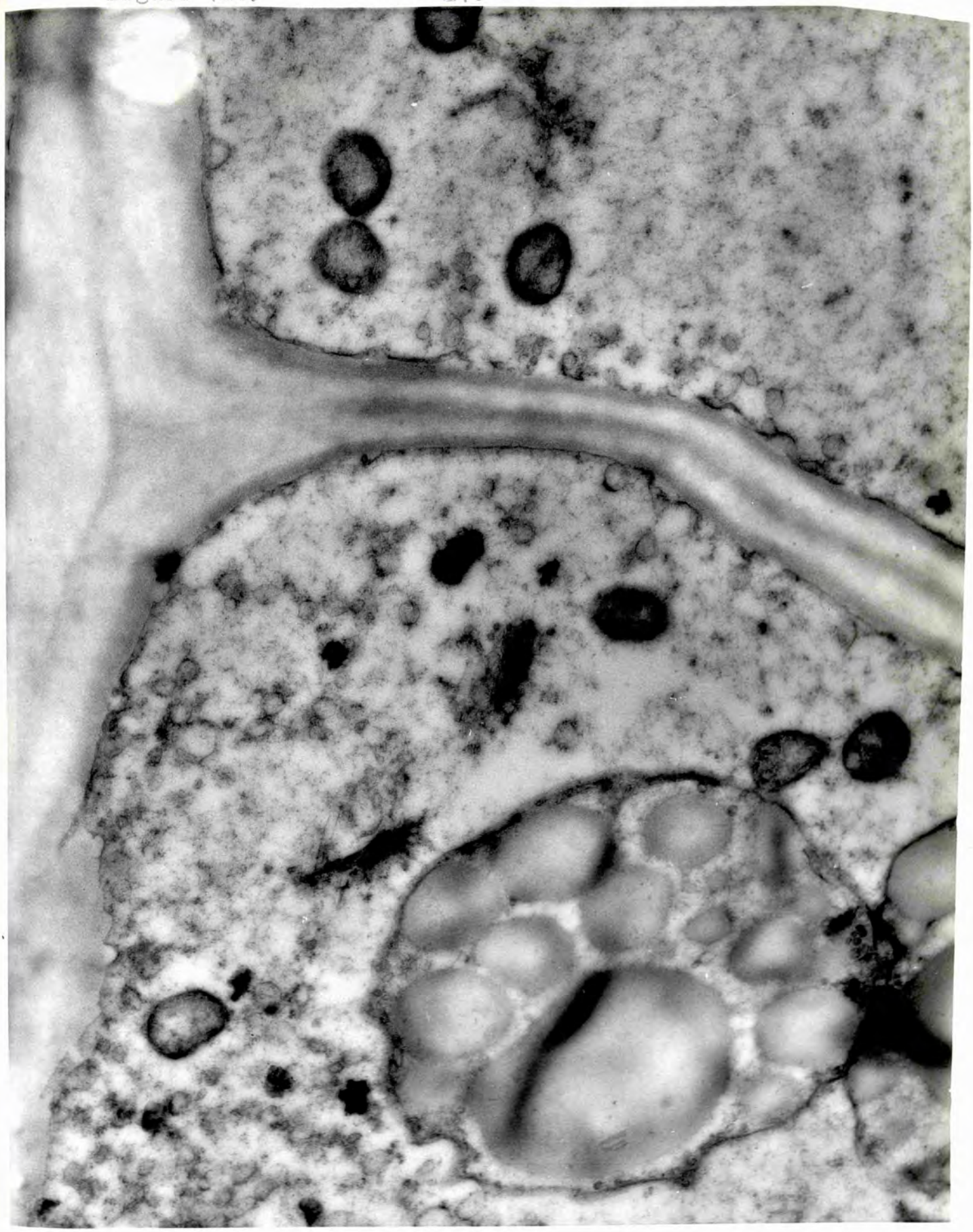
Sometimes it was possible to see concentric electron-dense regions in the larger starch grains in the cells near the periphery of the root-cap. The amount of staining of the starch grains by $KMnO_4$ could not be regulated; using exactly the same fixing procedure starch grains in some roots would be electron dense, while those in others would be transparent to electrons. The size of amyloplasts ranged from 1.0 -3.0 μ across the widest diameter.

Figure 41: Amyloplasts x 15,000. $KMnO_4$. Siemens microscope





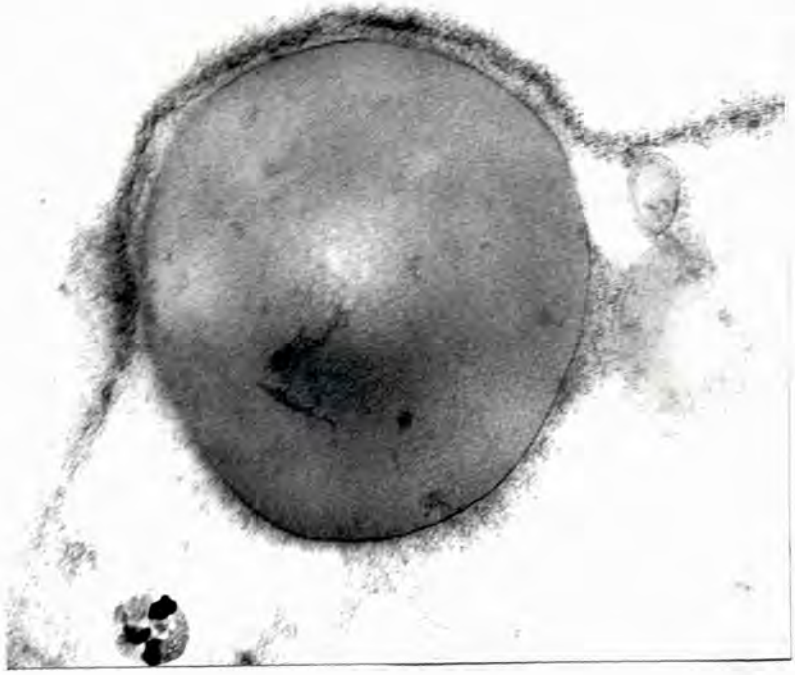
Amyloplasts: x 15,000. $KMnO_4$ Siemens microscope



Amyloplast: x 25,000. OsO_4 EM.6

In the high resolution electron micrographs there often appeared to be a very thin (30 Å) membrane around the individual starch grains.

Figure 42: x 40000. $KMnO_4$. EM.6. Starch grain.



The bounding membrane of the amyloplast was seen to be double in some sections, the dimensions and appearance being similar to that of the membranes of the endoplasmic reticulum. Frequently there were internal membranes continuous with the bounding membrane. These usually ran obliquely to the radius. The internal membranes had the same dimensions as the bounding.

The nuclear membrane stained in a similar way to the endoplasmic reticulum and had several pores in it, (Figure 43).

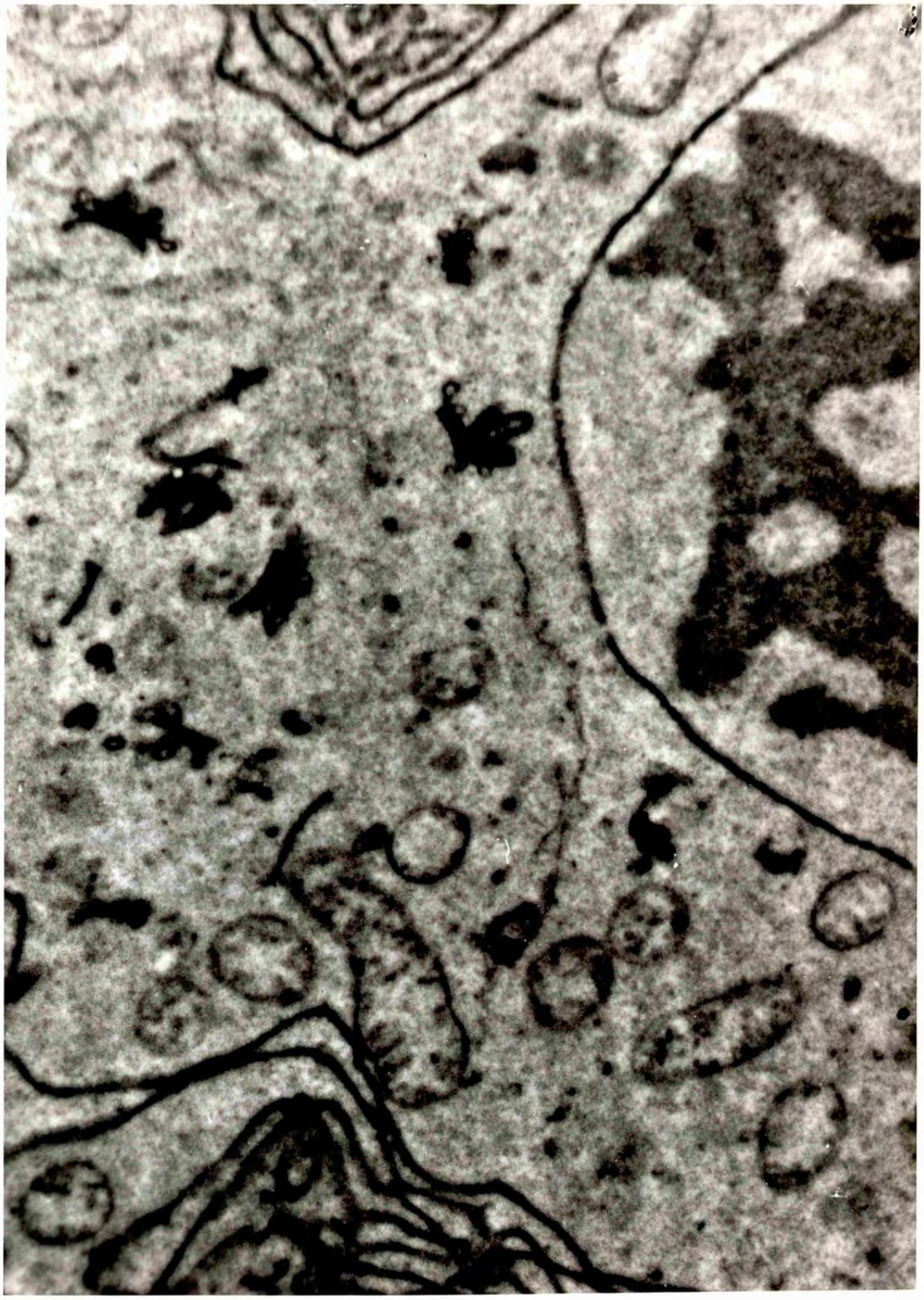


Figure 43: x 15,000

The pores were 200-400 Å in diameter. Sometimes there were larger discontinuities in the nuclear membrane, but this was more frequent in the meristem than in the root-cap cells.

The nucleoplasm had the same granular appearance as the ground substance of the cytoplasm, whether it was fixed with $KMnO_4$ or OsO_4. The nucleoli were distinct because of their staining properties. They were usually circular in cross section and sometimes contained numerous small vacuoles. No membrane could be seen around the nucleolus.

The plasma membrane was demonstrated as a single layered structure when $KMnO_4$ was used as a fixative.

Vacuoles and lipid bodies occurred abundantly in the meristem and promeristem cells, but were rare in root-cap cells. Apart from density of contents the vacuoles and "lipid" bodies of the meristematic cells were alike in appearance but the vacuoles were rather larger than the lipid bodies. Vacuoles were seen in the outer root-cap cells and lipid bodies were not seen in the root-cap. Both structures were limited by a single membrane. (Figures 25 and 26.)

The cell wall showed little in the way of structure. It was transparent to electrons, except occasionally along the region where one would expect the middle lamella to be. Plasmadesmata interrupted the wall at frequent intervals. The great majority of plasmadesmada were confined to the transverse walls - there were very few on the walls which were parallel to the long axis of the root. (See Figures, 33, 35 and 44.)

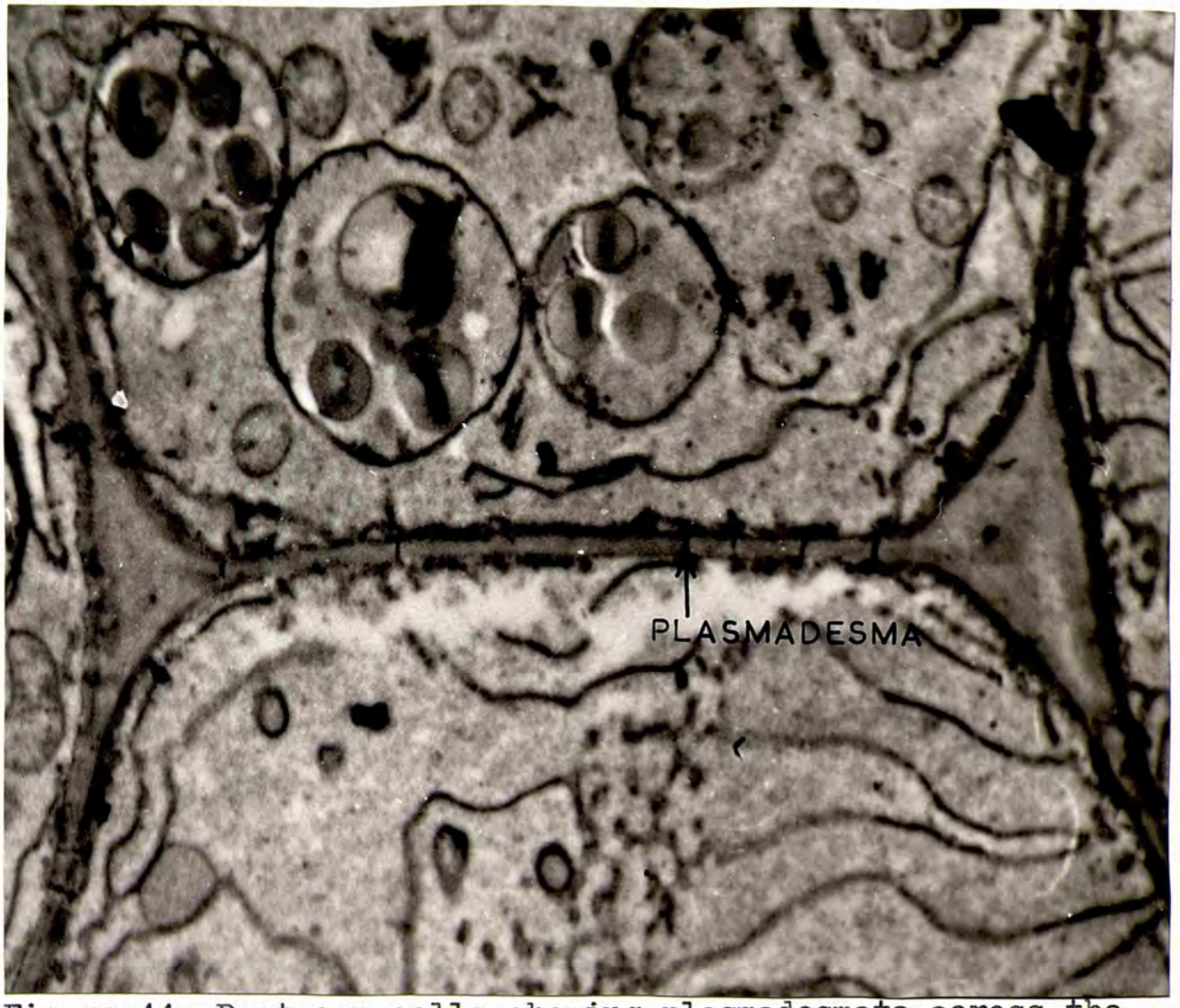


Figure 44: Root-cap cells showing plasmadesmata across the transverse wall, plastids at the distal end of the upper cell and endoplasmic reticulum at the proximal end of the lower cell. Fixed with $KMnO_4$ x 10,000. Siemensmicroscope.

G. The effect of gravitational stimulus on the ultrastructure of the root-cap cell.

Two types of change in the cell ultrastructure as a result of gravitational stimulus were looked for. The first was the redistribution of cell components following re-orientation of the plant organ. This would occur if the cell component were of a different density from the surrounding cytoplasm.

The second type of change which might be detectable was modification of the cell ultrastructure visualised by changes in staining properties or in granularity. It was expected that if such changes occurred, it would be a secondary reaction resulting either directly or indirectly from movement of cell components, since gravity can act directly only upon mass causing redistribution of bodies in fluid of different density: under the influence of gravity particles of lower density than the surrounding protoplasm would rise to the top of the cell while those of higher density would sediment. This is the only way whereby the influence of gravity can be directly made manifest in the cell.

The only change observed in staining properties and granularity of the cell following horizontal stimulation was the one already recorded (page 126) and later shown to be an artefact. No histochemical or cytochemical tests were applied to the tissue. Such tests might well prove very valuable in the study of the effect of gravity on plant cells especially now that the localisation of specific enzyme activity is becoming possible by the combined use of cytochemical tests and the electron microscope.

(i) Redistribution of cell components.

If the redistribution of any cell component does trigger off the chain reactions leading to geotropic curvature, it must occur within the first minute after re-orientation of the organ since such a short stimulation time will lead to curvature. Amyloplasts have been shown to move sufficiently rapidly. The following experiments were designed in order to detect whether other cell components move under the influence of gravity.

(a) Method: Straight roots of Vicia faba 2-3 cm. long were selected; after an initial two hours equilibration in a normal position, they were turned through 90°

so that their roots were horizontal, and left for 20 minutes. A tangential cut was made so that the orientation of the roots could be easily determined (see page 98) and the roots were immediatly immersed in the fixative, maintaining the horizontal orientation.

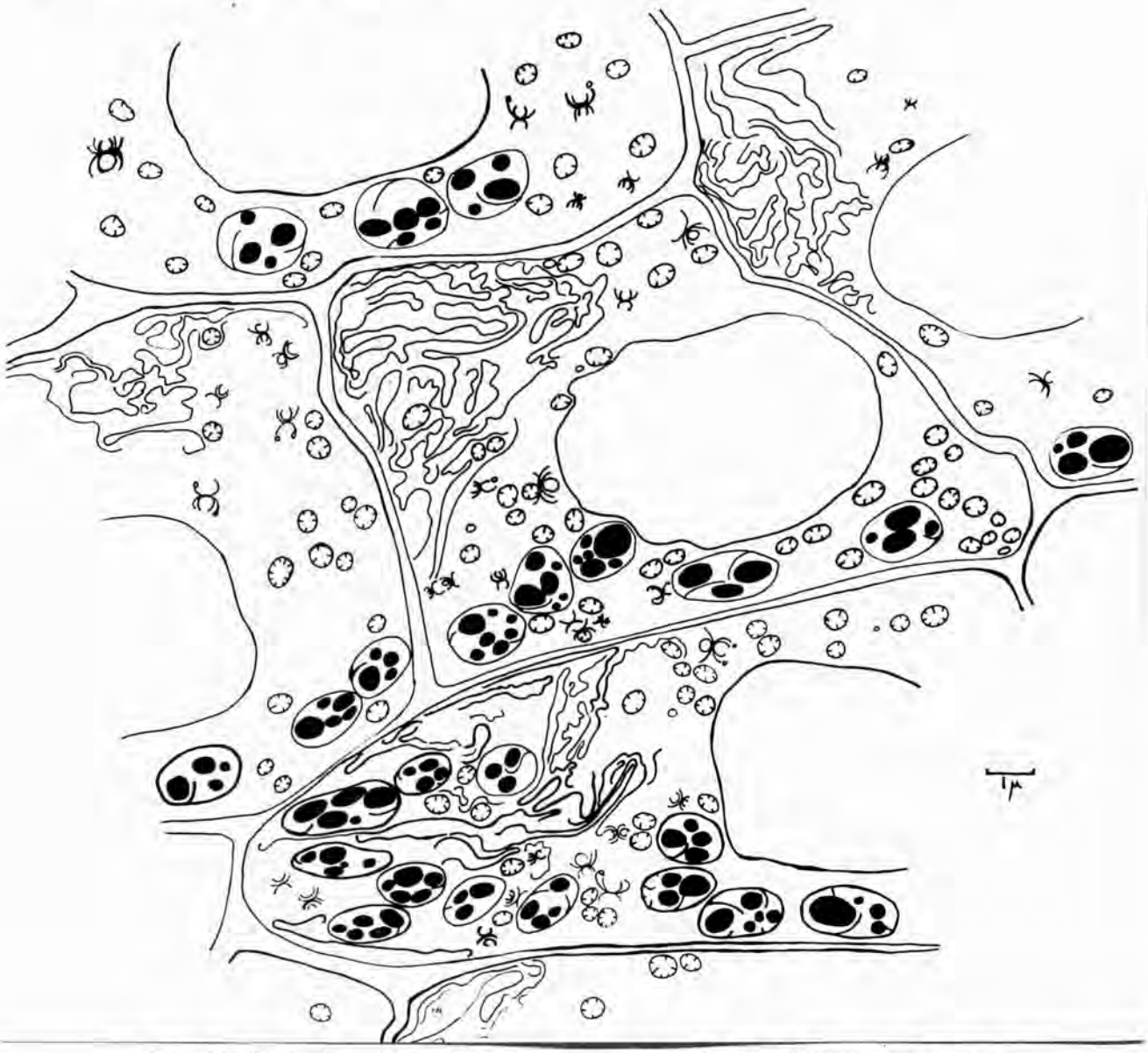
Photographs were taken of as many complete cells as possible from each of eleven root-caps shown below:

Root	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	Total
No. of cells observed	14	4	6	3	3	4	7	8	8	8	5	70

Since the field for photographing was restricted by the microscope, it was necessary to take several photographs to cover each cell. The negatives were then projected with constant magnification through a 35 mm. photographic enlarger onto typewriting paper of uniform weight. The uniformity of the ream of paper used was tested by weighing every tenth sheet. A composite outline drawing was made with the nucleus endoplasmic reticulum, mitochondria, dictyosomes and

amyloplasts drawn in. For each cell the direction of gravitational stimulus was recorded.

Figure 45: Composite drawing made from electron micrographs of cells from a root which had been horizontal for 20 minutes.



In order to divide each cell into four sectors of equal area along its axis and along the axis at right angles to the long axis (i.e. in the direction of the pull of gravity during stimulation), each cell

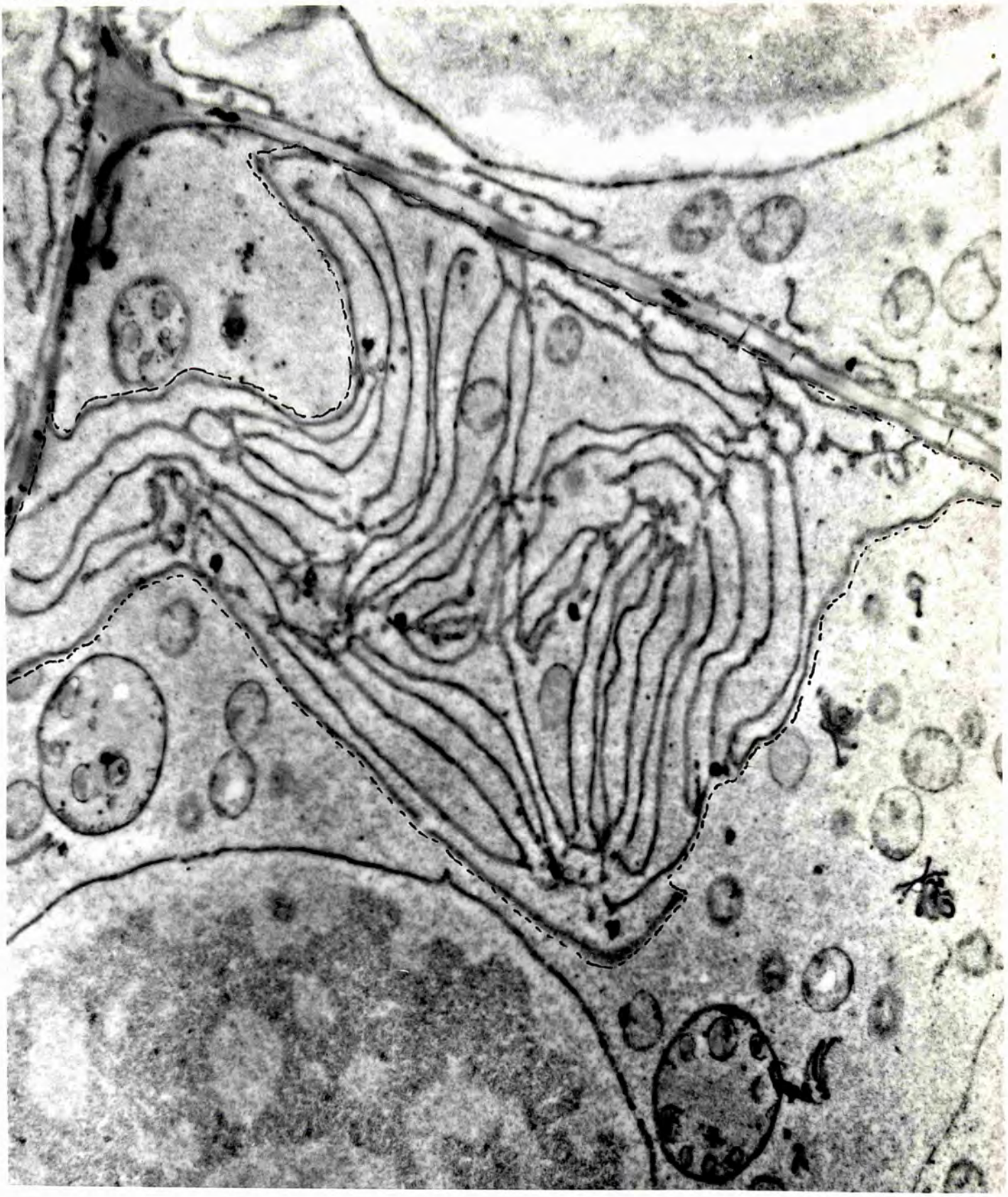
drawing was cut along the cell-wall/cytoplasm boundary. The drawing was then suspended at a point at its periphery by a pin holding a plumb-line so that the plumb-line hung parallel to the direction of the long axis of the root. If the "cut-out" is freely suspended in this way then the plumb-line will bisect it into two exactly equal areas. The cell was cut in half along this line. Each half was then suspended freely at its periphery so that the plumb-line hung at right angles to the first division. Thus each half 'cell' was divided into two halves of equal area, the division being in the direction of the pull of gravity during stimulation. The four sectors were labelled A, B, C and D systematically, as shown in Figure 47 page 166a. Each of the four sectors should have been equal in weight and therefore in area and in the volume which they represented, to each of the others.

The numbers of plastids, mitochondria and dictyosomes in each of the four sectors was counted.

It was decided that since sectors A, B, C and D differed in size from cell to cell, distribution of organelles represented as a number per sector would not be so informative as a measure of concentration.

It was therefore necessary to have a measure of the relative sizes of the sectors, and an accurate and convenient way for determining these was by weighing each of the sectors on a spring torsion balance. Assuming that the sections which the drawings represented were of uniform thickness, the weight of the sector was proportional to the volume of cytoplasm which it represented. Within the limits of the accuracy of the other stages in the experiment, it was safe to make this assumption since all sections showed silver interference colours and were in all probability between 65 and 85 milli-micron thick. This gives a possibility for only a small error compared with the error involved in the drawing and cutting out of the drawings.

The initial weighings made it possible to calculate the frequency of each type of organelle in each of the sectors. A further set of weighings was made inorder to determine the distribution of dictyosomes and mitochondria in the cytoplasm. For these the areas occupied by plastids and nuclei were cut away, and the remaining part of the sector was weighed. Besides this, the weight of any large mass of endoplasmic reticulum occurring in any one of the



Photograph to show how the endoplasmic reticulum was cut out (page 158). The area enclosed by the dotted line was cut from the drawing and weighed.

$KMnO_4$. Siemens.

sectors was recorded to reveal whether there is, as suspected, a significantly higher concentration of endoplasmic reticulum in stimulated cells along the wall opposite the amyloplasts (see Figure 46).

Methods of statistical analyses.

For making an analysis of variance of the results it was necessary to use populations of results which were normally distributed. The distributions of the populations of numbers of mitochondria, dictyosomes and endoplasmic reticulum within the sectors were tested for normality and formulae for transforming non-normal distributions to normal ones were looked for, with the following results:

	Distribution	Transformation
No. of mitochondria/sector	Not normal	$\sqrt{x}$
No. of dictyosomes/sector	Not normal	$\mathrm{Sin}^{-1}\sqrt{\frac{x}{X}}$ where x is the greatest no. of dictyosomes observed in any one sector (21)
Amt. of endoplasmic reticulum/sector	Not normal	None was found
No. of mitochondria/unit vol. of cytoplasm	Normal	-
No. of dictyosomes/unit vol. of cytoplasm	Normal	-
Amt. of endoplasmic reticulum/unit vol. of cytoplasm	Not normal	None was found

Since a suitable formula could not be found to transform the data for the amount of endoplasmic reticulum to give a better approximation to a normal distribution than the original data, the original data was used.

An analysis of variance was run on the measurements. This led to an estimate of the between-roots variance, the between-cells-within-roots variance, the between-sectors variance and the root x sector interaction. The model for the analysis of variance table is given below.

Source of Variance	Degrees of Freedom	Components of Variance
Between roots (R)	$r-1 = 10$	$\sigma_0^2 + s\sigma_C^2 + s\beta\sigma_R^2$
Between cells within roots (C)	$\sum_{r=1}^{11} (c-1) = 59$	$\sigma_0^2 + s\sigma_C^2$
Between sectors (S)	$s-1 = 3$	$\sigma_0^2 + \beta\sigma_R^2 + \beta\sigma_S^2$
Root x sector interaction (RS)	$(r-1)(s-1) = 30$	$\sigma_0^2 + \beta\sigma_{RS}^2$
Residual	177	
Total	279	

In this table β represents a factor dependent on the number of cells measured in each root, r represents the number of roots, c represents the number of cells from which counts were made and s represents the number of sectors in each cell.

The analysis of variance tables shown in Figure 48 show the results which are summarised in Figure 47 (Page 167)

The individual sector means for each of the eleven roots, the over-all means for each root and the over-all sector means are given in the appendix (page 207). The individual sector means for the eleven roots are also shown with their fiducial limits in the diagrams of Figures 49 and 50 (pp 181 & 182). The fiducial limits were calculated from the formula

Least significant difference (i.e. fiducial limits) =

$$\frac{\text{Residual standard deviation}}{\sqrt{n}} \times \sqrt{2} \times t$$

where n is the number of cells recorded from the root under consideration and where t is 1.98 (i.e. t for 85 degrees of freedom at the 0.05 significance level).

Further statistical tests were carried out in order to determine whether there was any degree of correlation between mitochondria, dictyosomes and endoplasmic reticulum.

(c) Results of statistical analyses

Analysis of Variance: In making an analysis of variance the components which had to be taken into account were those due to the variation between the eleven roots from which the counts of cell organelles were taken, variation between the cells within the roots and the variation between the four sectors into which each cell was systematically divided. The root x sector interaction was also taken into consideration. The results of the analysis are shown below:

FIGURE 48

	VARIANCE COMPONENT							
	Between Roots		Between Cells Within Roots		Between Sectors		Root x Sector Interaction	
	F. ratio	Signi-ficance	F. ratio	Signi-ficance	F. ratio	Signi-ficance	F. ratio	Signi-ficance
No. of mito-chondria	2.20	0.05	1.3	0.05	1.65	N.S.	1.2	N.S.
No. of dictyo-somes	1.28	N.S.	1.51	0.05	3.09	0.05	1.37	N.S.
Area of sector occupied by endoplasmic reticulum	<1	N.S.	1.36	N.S.	1.80	N.S.	1.75	0.05
No. of mito-chondria/unit vol.cytoplasm	1.43	N.S.	1.53	0.05	<1	N.S.	1.61	0.05
No. of dictyo-somes/unit vol. of cytoplasm	<1	N.S.	1.36	N.S.	<1	N.S.	1.77	0.01
Vol. of unit vol. of cytoplasm occupied by endoplasmic reticulum	1.13	N.S.	1.21	N.S.	<1	N.S.	2.46	0.001

N.S. = Not Significant

Wherever a unit volume of cytoplasm is referred to, the unit is equivalent to approximately 6 ~~x 10^9~~ cubic microns. This value is the volume of cytoplasm represented by 100 mg. of the paper used for making the drawings of the cells. It was calculated in the following way:

1.693 micron were represented in the drawings by 7.1 mm. (1/15,000 inch)

$$\therefore \quad 1 \text{ cm.} \equiv \frac{1.693}{0.71} \mu$$

$$= 2.384 \mu$$

$$\therefore 1 \text{ cm.}^2 \equiv 5.683 \mu^2$$

A rectangular piece of paper $(20.4 \times 25.5)\text{cm}^2$ weighed 3.713 grams

$$\therefore \quad \frac{520.2}{3.713} \text{ cm}^2 \equiv 1 \text{ gram}$$

$$\therefore \quad \frac{52.02}{3.713} \text{ cm}^2 \equiv 100 \text{ mg.}$$

$$\therefore \quad 14.01 \text{ cm}^2 \equiv 100 \text{ mg.}$$

$$\therefore \quad 79.62 \equiv 100 \text{ mg.}$$

The sections represented by the drawing were 75 mμ thick.

$$100 \text{ mg} \equiv 79.62 \times 75 \times 10^{-3} \mu^3 = 5971 \times 10^{-3} \mu^3$$

100 mg. of paper represent 5.97 μ^3 of cytoplasm.

Taking the 0.05 level of significance, the variance ratio for the number of mitochondria in different roots indicated that the number of mitochondria in a cell was dependent on the root from which the cell was taken. The other cell organelles did not show this difference from root to root.

The number of mitochondria and the number of dictyosomes varied from cell to cell. This variation was probably due to the differences in the level of the cell from which the section came: a cell-section cut through the middle of its nucleus would probably contain less mitochondria and dictyosomes than an equally large cell-section which did not contain part of the nucleus.

The number of mitochondria per unit volume of cytoplasm varied from cell to cell. This variation might be caused by variation in the relative position of the cell in the root-cap: perhaps more peripheral cells contain a higher (or lower) concentration of mitochondria than the central ones. Dictyosomes did not show any variation in concentration from cell to cell.

Neither the amount nor the "concentration" of endoplasmic reticulum show significant differences from root to root and from cell to cell.

The significant root x sector interactions are very important. They occur in measurements of concentration in the cytoplasm of mitochondria and dictyosomes. They also occur in the measurements of the amount and the concentration of endoplasmic reticulum in the cytoplasm. Such interactions imply considerable variation in sedimentation or creaming of organelles or in displacement of cytoplasm from root to root. This interaction indicates the variability in the material: unilateral distribution of components observed in one root will not necessarily occur in the same way in another root. Root XI for example (see Page 181,182) was particularly abnormal when compared with the other roots. This variability from root to root, and the occurrence of "abnormal" roots will mask slight movement of cell organelles.

Except for the number of dictyosomes per sectors, the variance ratios between the sectors are very small for measurements of the numbers and concentration of the cell organelles indicating that the differences between sectors is no larger than might be expected from the material. Therefore no special difference in numbers of mitochondria and endoplasmic reticulum is established between the sectors, nor does the

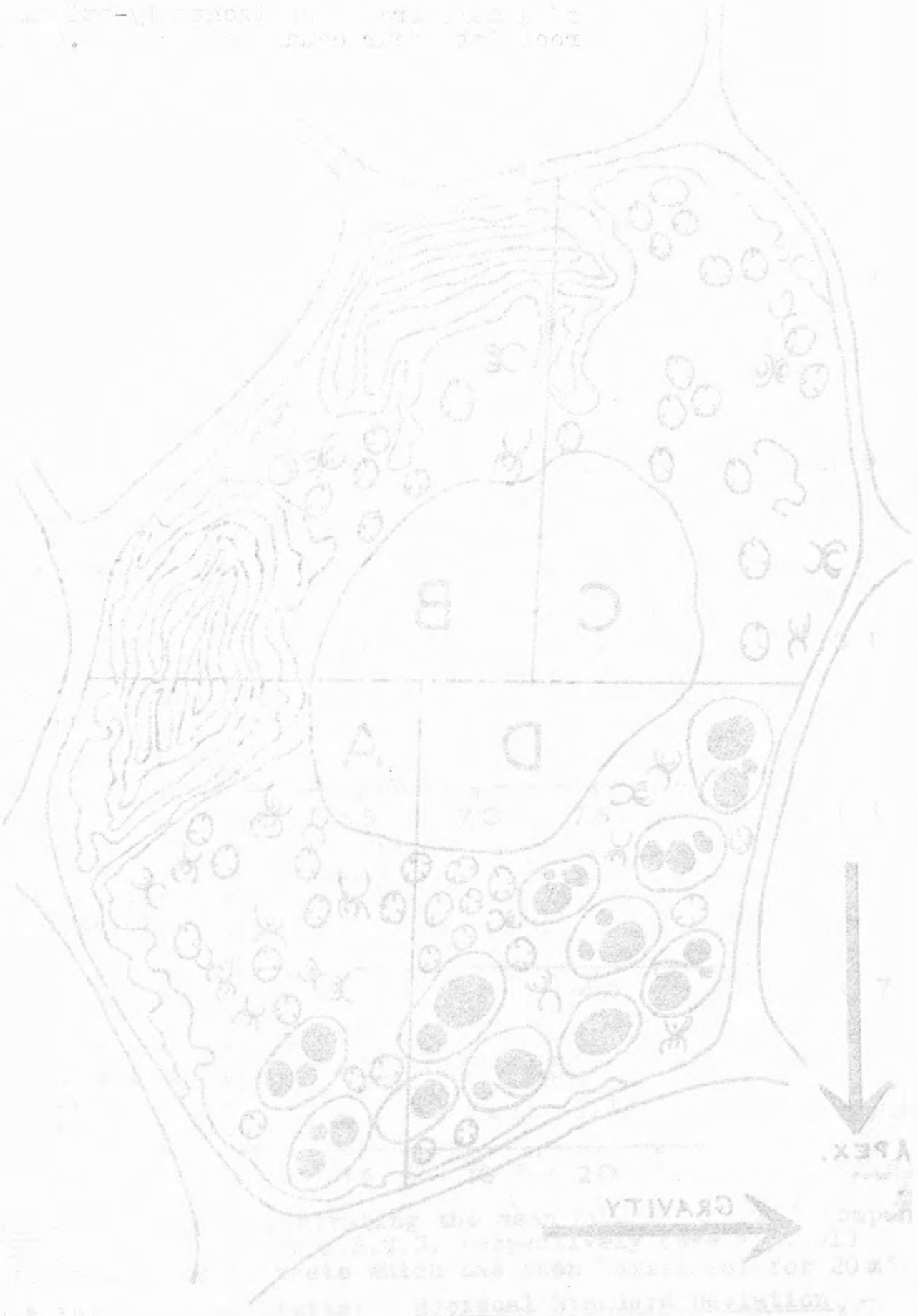
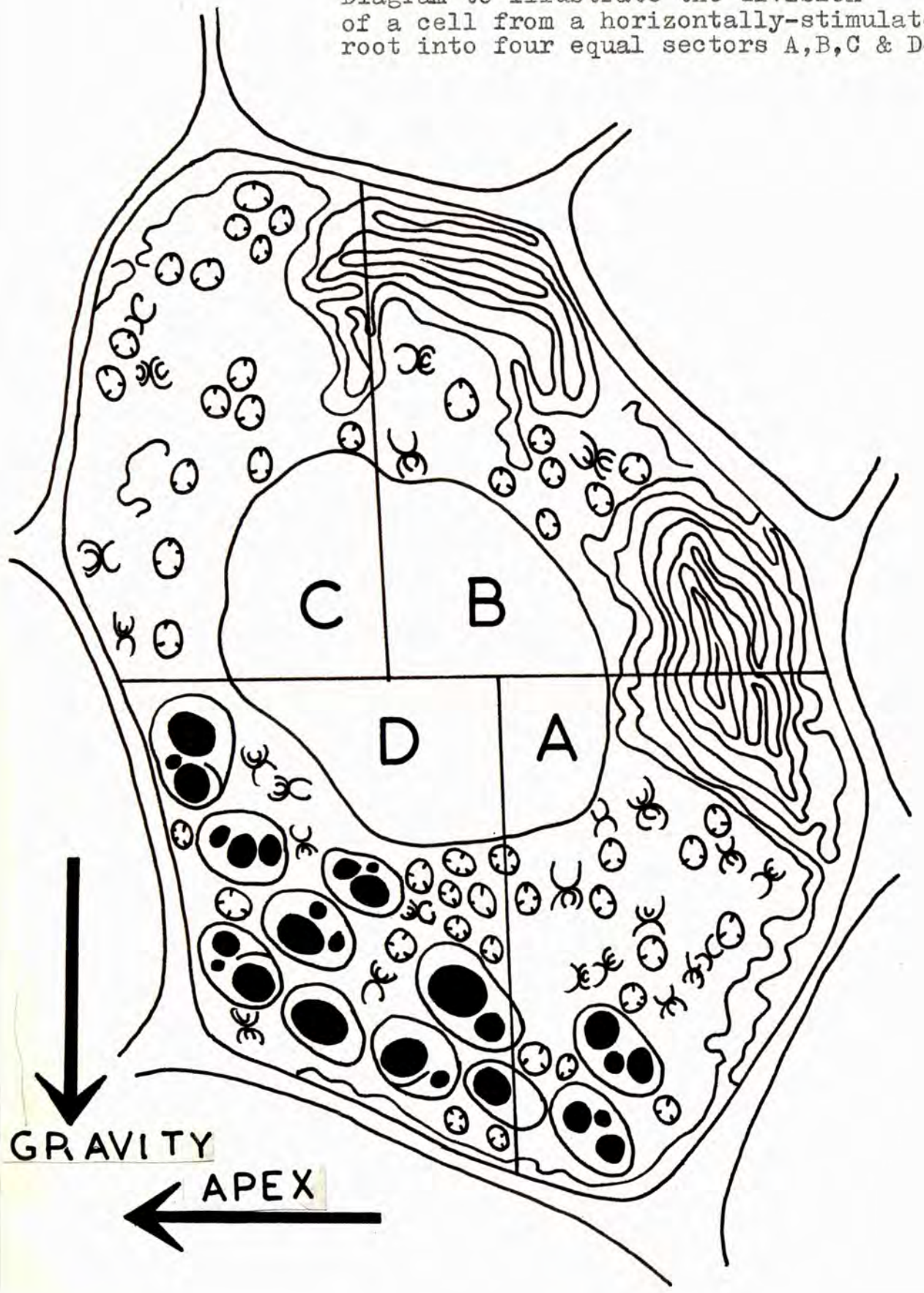

B
C
A
D
APEX
GRAVITY

Diagram to illustrate the division of a cell from a horizontally-stimulated root into four equal sectors A,B,C & D.



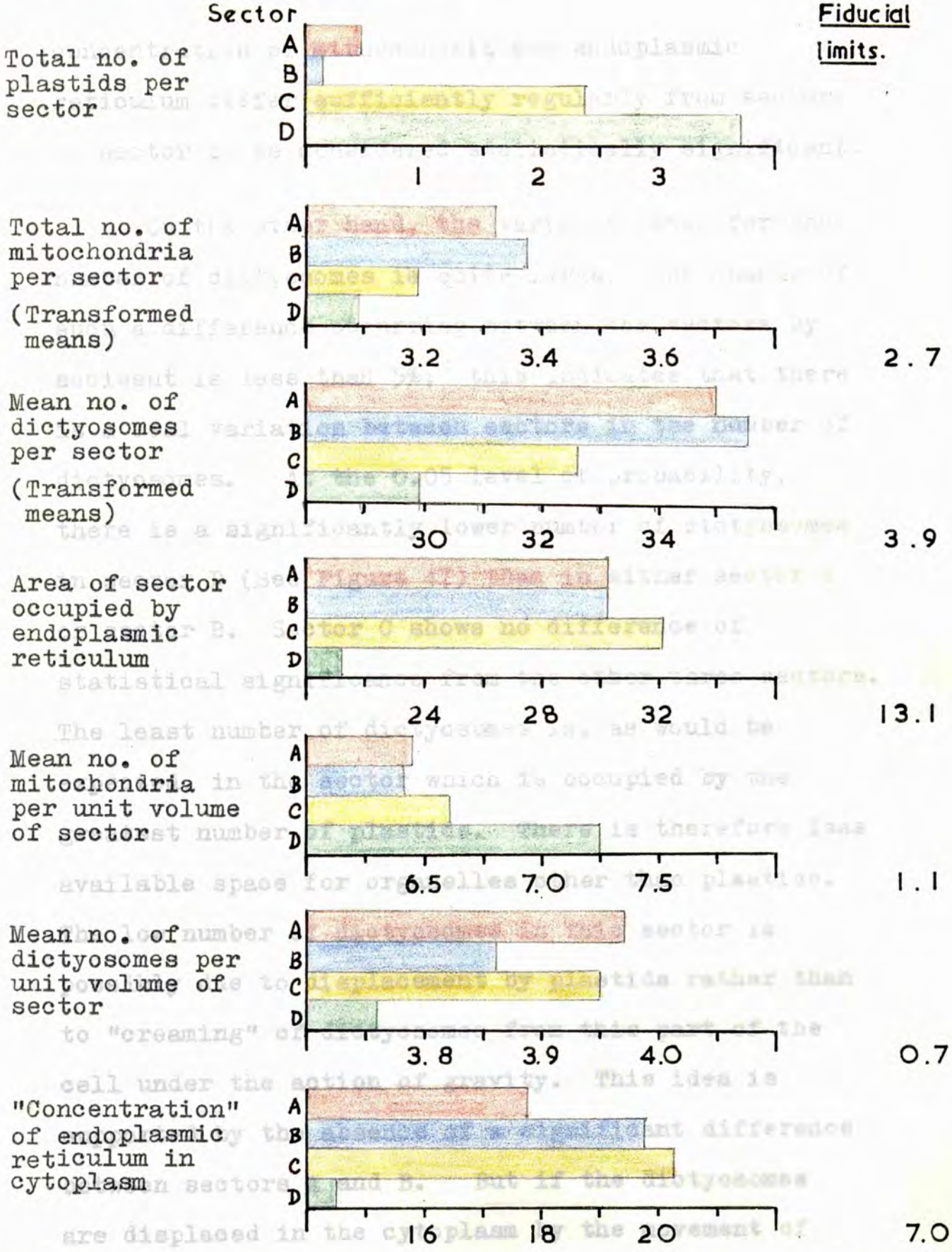


FIGURE 47: Diagram illustrating the mean numbers of cell components recorded in sectors A,B,C,D, respectively (see Fig. 51) from 70 cells from 11 roots which had been horizontal for 20 min.

Formula for fiducial limits: $\frac{\text{Residual Standard Deviation}}{\sqrt{n}} \times \sqrt{2} \times t.$

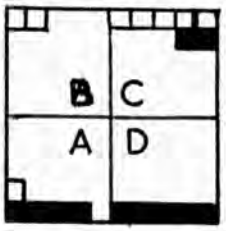
concentration of mitochondria and endoplasmic reticulum differ sufficiently regularly from sector to sector to be considered statistically significant.

On the other hand, the variance ratio for the number of dictyosomes is quite large; the chance of such a difference occurring between the sectors by accident is less than 5%; this indicates that there is a real variation between sectors in the number of dictyosomes. At the 0.05 level of probability, there is a significantly lower number of dictyosomes in sector D (See Figure 47) than in either sector A or sector B. Sector C shows no difference of statistical significance from the other three sectors. The least number of dictyosomes is, as would be expected, in the sector which is occupied by the greatest number of plastids. There is therefore less available space for organelles other than plastids. The low number of dictyosomes in this sector is possibly due to displacement by plastids rather than to "creaming" of dictyosomes from this part of the cell under the action of gravity. This idea is supported by the absence of a significant difference between sectors A and B. But if the dictyosomes are displaced in the cytoplasm by the movement of

the plastids, why do the mitochondria not show a similar displacement? The dissimilarity between the distribution of these two organelles suggests that the dictyosomes are "creaming" under the action of gravity. However, if one looks at the patterns shown by the mean values for the numbers of mitochondria and dictyosomes (Figure 47) in the four sectors, the trend which is significant for the dictyosomes is present for mitochondria, although it does not attain statistical significance at the 0.05 probability level. Consideration of the mean values for the individual roots shows that although statistical differences at the 0.05 probability level are seldom attained, and that where there are significant differences at this level the pattern from root to root is rather different, the one consistent character of the roots showing significant differences between the sectors is that there are always less mitochondria in sector D. This indicates a similar behaviour of mitochondria to that which attains statistical significance in the overall means for dictyosomes. It seems possible therefore that the starch grains do displace cytoplasm containing some of the dictyosomes and mitochondria to the upper half of the cell. This displacement could explain

the differences observed between sector D and the other sectors. This conclusion is supported by the absence of any sign of a tendency for sector D to contain more or less mitochondria or dictyosomes per unit volume of cytoplasm. If dictyosomes or mitochondria were "creaming" there would be a difference between the upper and lower sectors in both number and concentration. The observed differences in numbers but not concentration imply mass movement of cytoplasm caused probably by displacement by plastids.

Endoplasmic reticulum: The average amount of endoplasmic reticulum in sector D is lower than the amount in each of the other three sectors on both a per-sector basis and on a per-unit-volume-of-cytoplasm basis, but the difference is not large enough to be regarded as statistically significant. Where differences in individual roots are significant there is always less endoplasmic reticulum in sector D. In these roots with significantly less in sector D, there is always significantly more in sector C.

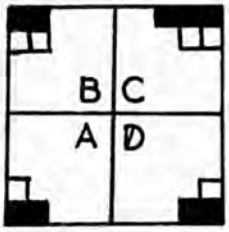


represents 2 roots showing significantly more endoplasmic reticulum in the sector indicated

represents 2 roots showing significantly less endoplasmic reticulum in the sector indicated

The low amount of endoplasmic reticulum in D can be explained possibly by displacement by plastids, but it is difficult to explain the low amount in A in such a way.

When the amounts of endoplasmic reticulum are calculated on the basis of the proportion of cytoplasm occupied by endoplasmic reticulum the picture is not so clear and fewer roots show significant differences.



represents 2 roots showing significantly more endoplasmic reticulum in the sector indicated

represents 2 roots showing significantly less endoplasmic reticulum in the sector indicated

There are still more roots which show significantly less endoplasmic reticulum in sectors A and D than in sectors B and C, but there are more roots which show significantly less endoplasmic reticulum in sectors B and C than in sectors A and D.

The amount of endoplasmic reticulum in the upper half of the cell was compared with the amount in the lower half by adding the individual values for B to those for C, and the values for A to those for D. An analysis of variance was performed on the results with the following results

FIGURE 52:

Source of variance	Sum of Squares	d.f.	Mean Square	F. ratio
Between roots	30,355	10	3,036	<1 N.S.
Between sectors	15,943	1	15,943	2.23 N.S.
Between cells within roots	71,495	10	7,149	$2.71 > 100/1$
Root x sector interaction	228,803	33	6,933	$2.63 > 100/1$
Residual	224,162	85	2,637	
Total	570,759	139		

Residual Standard Deviation $= \sqrt{2,637.20} = 51.35$

Root x Sector Interaction $\sqrt{\frac{7149.49 - 2,637.20}{6.36}} = 26.64$

The standard error of the difference between two means $= \sqrt{\frac{7,149.49 \times 2}{70}}$

$= 14.29$

At the 0.05 probability level it would be necessary to observe a difference between the mean values of 1.96 x 14.29 = 28.01. The difference observed was actually 21.35, the mean value for (A + D) being less than the mean value for (B + C). This difference cannot be regarded as statistically significant at the 0.05 probability level. Of the 11 individual roots, three had significantly more endoplasmic reticulum in the upper half of the cell as shown in the table below.

FIGURE 53a:

Root	No. of cells	(A+D) Lower	(B+C) Upper	Mean(A+D) = L	Mean(B+C) = U	Difference between means U-L	Least significant difference	Significance at 20/1
I	14	477	1122	34	80	+46	39	Less in (A+D)
II	4	234	387	58	97	+39	72	
III	6	564	423	94	70	-24	59	
IV	3	0	295	0	99	+99	83	Less in (A+D)
V	3	195	291	32	65	+33	83	
VI	4	260	275	65	69	+4	72	
VII	7	393	616	56	88	+32	54	
VIII	8	608	316	76	39	-37	51	
IX	8	576	955	72	119	+47	51	
X	8	595	214	74	27	-47	51	
XI	5	34	533	7	107	+100	64	Less in (A+D)
Total	70	3837	5331	54.81	76.16	+21.35	28.01	

Similar analyses were carried out for the proportion of cytoplasm occupied by endoplasmic reticulum in the two halves, with the following results:

FIGURE 53b:

Root	No. of cells	(A+D) Lower	(B+C) Upper	Mean(A+D) = L	Mean(B+C) = U	Difference between means U-L	Least significant difference	Significance at 20/1
I	14	1.381	3.097	0.099	0.221	+0.122	0.103	Less in (A+D)
II	4	0.615	1.052	0.1538	0.263	+0.1092	0.1900	
III	6	1.287	0.808	0.2146	0.1347	-0.0799	0.1570	
IV	3	0	0.729	0	0.243	+0.243	0.222	Less in (A+D)
V	3	0.296	0.543	0.0987	0.181	+0.1023	0.222	
VI	4	0.602	0.509	0.1505	0.1272	-0.0233	0.1900	
VII	7	1.027	1.446	0.1467	0.2066	+0.0599	0.145	
VIII	8	2.077	0.884	0.2596	0.1105	-0.1491	0.137	Less in (B+C)
IX	8	1.556	2.895	0.1945	0.3619	+0.1674	0.137	Less in (A+D)
X	8	2.228	0.840	0.2785	0.105	-0.1735	0.137	Less in (B+C)
XI	5	0.177	2.044	0.0354	0.4088	+0.3734	0.1700	Less in (A+D)
Total	70	11.246	14.847	0.161	0.212	+0.051	0.0958	

Similar analyses were carried out for the proportion of cytoplasm occupied by endoplasmic reticulum in the two halves, with results as shown in Figure 53b on page 174.

The results indicate that there is a certain amount of displacement by the plastids of the endoplasmic reticulum in the root-cap cells. This only attains significance in three of the eleven roots. However, this difference is consistent and can be correlated with the orientation of the root: there is more endoplasmic reticulum in the upper halves of these three roots. The same tendency occurs in all but three of the other roots but does not attain statistical significance.

These results are very interesting in relation to the observations of Němec (1900, 1901) made during his survey of the statocytes of plants. In material fixed in chromacetic acid and stained in haematoxylin he claimed to have seen densely staining protoplasmic bodies of a lamellate and granular nature. His drawings illustrating these bodies and their position in the cell before and after horizontal stimulation show a remarkable similarity with the groups of parallel endoplasmic reticulum membranes observed using an

electron microscope. Němec suggested that the reaction sequence leading to curvature is initiated by an interaction of statolith starch with the lamellate and granular protoplasmic bodies which were displaced by starch during its movement through the cytoplasm.

The results of these electron microscope studies suggest displacement of the endoplasmic reticulum by the movement of the plastids as do the results of Němec. It seems quite likely that Němec's protoplasmic bodies were no artefacts, but were masses of endoplasmic reticulum. Professor Mollenhauer has observed large whorled masses of endoplasmic reticulum in root-cap cells and he believes them to be fixation artefacts. These whorls of endoplasmic reticulum, in contrast to the parallel membranes observed here, occur mainly in the peripheral cells of the root-cap and bear no special relation to the orientation of the root with respect to gravity. They are probably not comparable with the endoplasmic reticulum measured in this work. The regularity of the membranes and their continuity suggests that they are not fixation artefact.

The mean value for the concentration of endoplasmic reticulum is higher in the upper halves of four of the roots than in the lower halves. It is lower in the

upper halves than the lower halves in two roots, both of which also had less (but not significantly so) endoplasmic reticulum in the upper halves on a quantitative basis. This increase in concentration implies either that the endoplasmic reticulum is displaced by the plastids to a greater extent than the cytoplasm containing mitochondria and dictyosomes, or that some new endoplasmic reticulum develops in the upper halves of the cells during horizontal stimulation. Němec observed the appearance and disappearance of the lamellate bodies during and after horizontal treatment, so if lamellate bodies were parallel masses of endoplasmic reticulum, Němec's observations suggest that endoplasmic reticulum will form quickly during geotropic stimulation. If this is so, the formation of endoplasmic reticulum at the upper side of the cell must reflect a gradient across the cell - possibly a chemical gradient or a gradient resulting from a potential difference across the cell - which leads to unilateral formation of endoplasmic reticulum.

(ii) Correlation of mitochondria, dictyosomes and endoplasmic reticulum with each other.

Mitochondria, dictyosomes and endoplasmic reticulum measurements have been correlated with each other. The correlation coefficients and significance levels are given in the table overleaf (page 178).

Sectors	Mitochondria correlated with dictyosomes	Mitochondria correlated with E.R.	Dictyosomes correlated with E.R.
A	r = .218 N.S.*	r = - .290 > 50/1	r = - .199 N.S.
B	r = .523 >1000/1	r = - .179 N.S.	r = - .354 > 100/1
C	r = .635 >1000/1	r = - .244 > 20/1	r = - .355 > 100/1
D	r = .475 >1000/1	r = - .198 N.S.	r = - .201 N.S.
Over all sectors	r = .482 >1000/1	r = - .206 > 20/1	r = - .254 > 100/1

	Mitochondria per 100 mg. of free cytoplasm correlated with dictyosomes per 100 mg. of free cytoplasm	Mitochondria per 100 mg. of free cytoplasm correlated with E.R. per 100 mg of free cytoplasm	Dictyosomes per 100 mg. of free cytoplasm correlated with E.R. per 100 mg. of free cytoplasm
A	r = .195 N.S.	r = - .376 > 100/1	r = - .252 > 20/1
B	r = .432 >1000/1	r = - .529 > 1000/1	r = - .562 > 1000/1
C	r = .412 >1000/1	r = - .476 > 1000/1	r = - .534 > 1000/1
D	r = .140 N.S.	r = - .089 N.S.	r = - .376 > 100/1
Over all sectors	r = .261 >100/1	r = - .323 > 1000/1	r = - .433 > 1000/1

* N.S. means not statistically significant taking p = 0.05 as the level of accepted significance.

The positive correlation between the total numbers of mitochondria and dictyosomes is a reflection of the uniform concentration between cells and the variation in cell size. The positive correlation between mitochondria and dictyosomes per unit volume is again a reflection of uniformity of concentration between cells and the variation observed here is due to varying thickness of the sections, so that apparent variation in concentration (i.e. mitochondria and dictyosomes increasing or decreasing together) is merely a reflection of variations in section thickness. If this is so, it is possible to calculate the approximate variation in section thickness from the variation in the numbers of mitochondria and dictyosomes. The mitochondria vary in number from 5.36 to 8.45 per unit volume of cytoplasm. The variation about the median value is 6.90 ± 1.54, i.e. 22%. A similar calculation for dictyosomes gives a variation of 3.79 ± 0.70, i.e. 19%. Taking the variation in mitochondria and dictyosomes as representing the variation in section thickness one gets a larger variation than was suggested earlier in the text (page 158) viz. 75 ± 10 mμ i.e. 13%, but it is still probably not much greater than the errors involved in projecting, drawing and

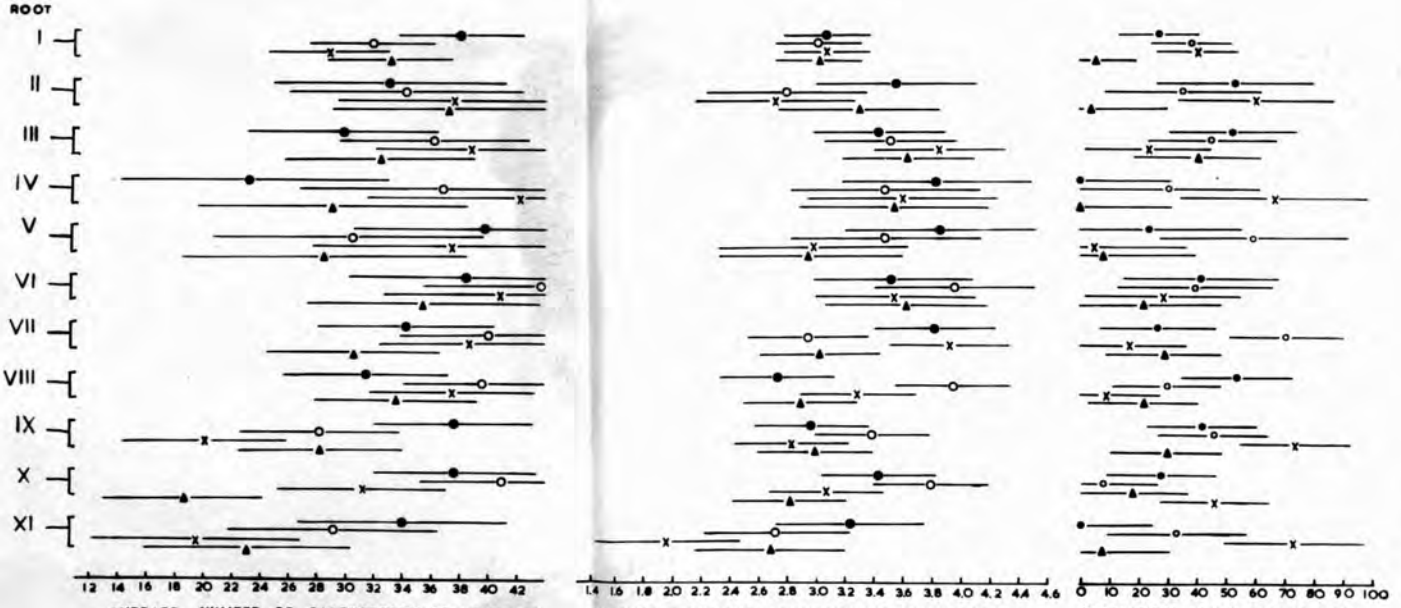
cutting out the cell outlines. Unlike the other errors, the variation in section thickness introduces a systematic error in the measurements from root to root since the cells recorded from one root were taken from one section or from a few consecutive sections from a ribbon of sections. The variation in cell thickness would therefore not be very great within one root, and would not lead to great differences from cell to cell. It would however cause marked differences from root to root. Section thickness should not markedly influence the differences between the sectors since the four sectors from one cell would in all probability be of the same thickness.

The correlation of both mitochondria and dictyosomes with endoplasmic reticulum is a negative one; as the amount of endoplasmic reticulum increases there is an accompanying fall in the number of mitochondria and dictyosomes. This negative correlation implies that the increase in endoplasmic reticulum in the upper half of the cell is caused by movement of the parallel endoplasmic reticulum membranes and displacement of the cytoplasm containing mitochondria and dictyosomes which are seldom seen amongst the endoplasmic reticulum membranes.

Figure 49

DIAGRAM ILLUSTRATING THE RESULTS OF THE EXPERIMENTS DESIGNED TO SHOW THE POSITION OF MITOCHONDRIA DICTYOSOMES AND ENDOPLASMIC RETICULUM IN THE ROOT-CAP CELL AFTER TWENTY MINUTES HORIZONTAL STIMULATION.

KEY TO SYMBOLS
- ● SECTOR A
- ○ SECTOR B
- X SECTOR C
- ▲ SECTOR D



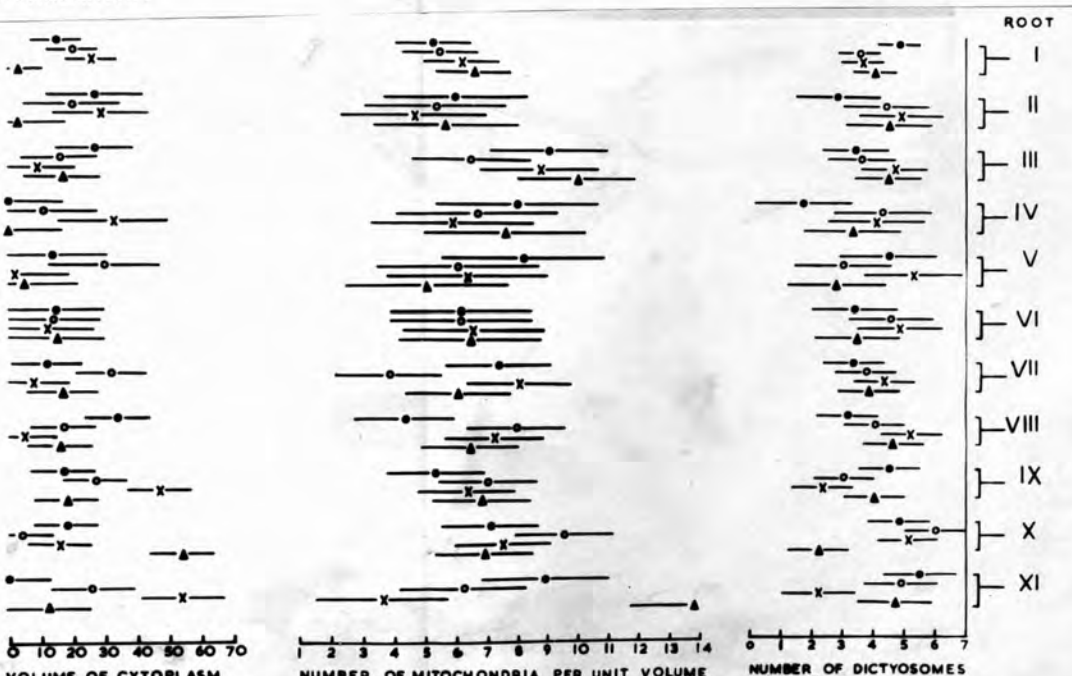
AVERAGE NUMBER OF DICTYOSOMES TRANSFORMED BY THE FORMULA $\sin^{-1}\sqrt{\frac{2x}{X}}$ WHERE X IS 21, I.E. THE GREATEST NUMBER IN ANY ONE SECTOR RECORDED.

AVERAGE NUMBER OF MITOCHONDRIA, TRANSFORMED BY THE FORMULA $\sqrt{x}$.

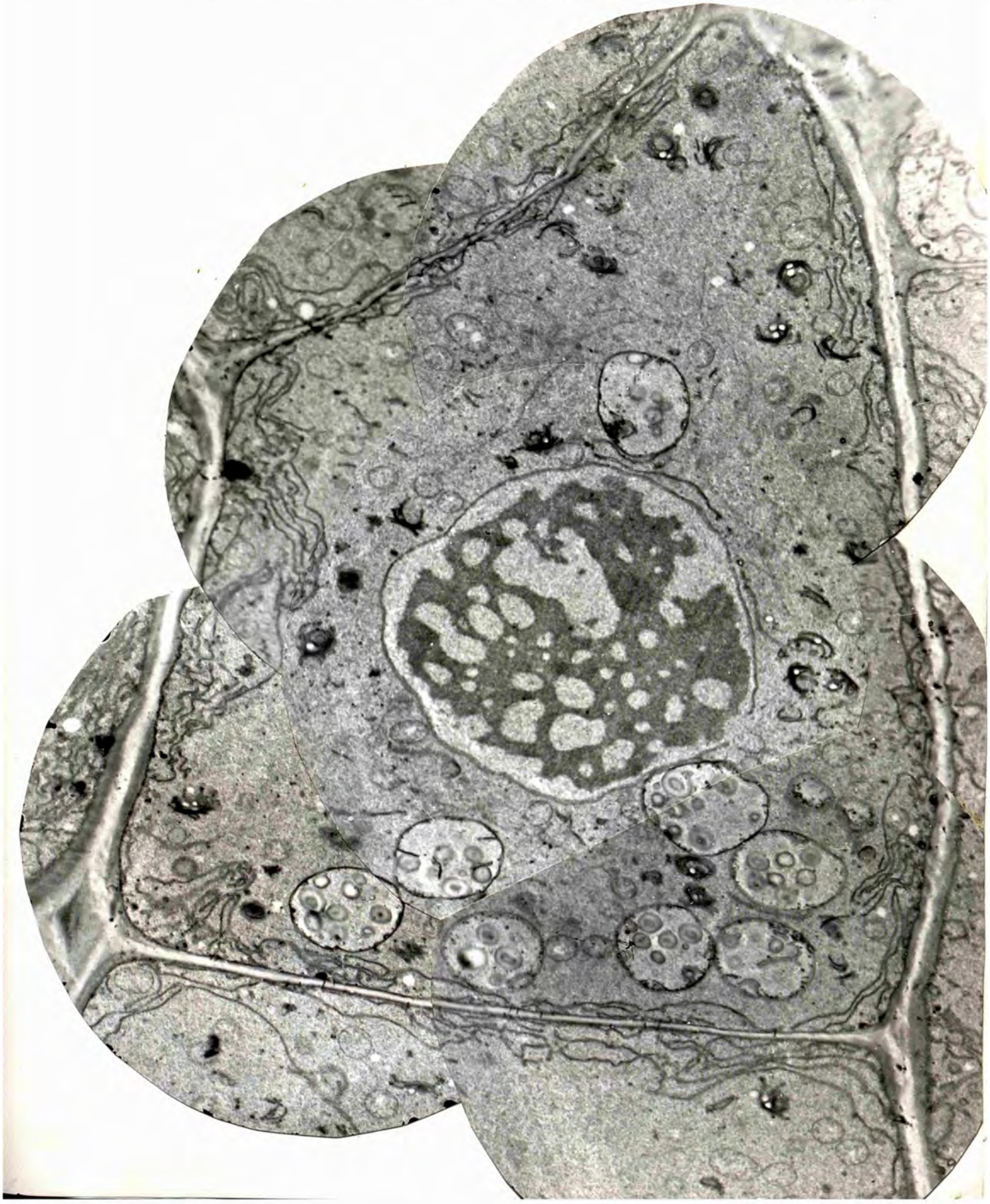
VOLUME OF SECTOR OCCUPIED BY ENDOPLASMIC RETICULUM.

DIAGRAM ILLUSTRATING THE RESULTS OF MEASUREMENTS OF THE CONCENTRATION DIFFERENCES IN RELATION TO GRAVITY OF MITOCHONDRIA DICTYOSOMES AND ENDOPLASMIC RETICULUM IN THE CYTOPLASM OF ROOT-CAP CELLS AFTER TWENTY MINUTES HORIZONTAL STIMULATION.

THE KEY TO SYMBOLS IS AS ON THE OPPOSITE PAGE



Complete root-cap cell. x 5,000. $KMnO_4$. Siemens.



CHAPTER IV

DISCUSSION

At the beginning of this work it was set out to do the following things:

1. Determine the speed of amyloplast sedimentation and compare it with the presentation time.
2. Correlate further than had been done previously the spatial distribution of amyloplasts during gravitational stimulation under various conditions with the subsequent geotropic bending reaction.
3. Discover (a) the effect of the movement of starch through the cytoplasm on the ultrastructure of the cell and

   (b) the direct effect of gravity on the ultrastructure of the cell.

It was hoped that a combination of these approaches would elucidate the mechanism of geoperception especially with respect to the statolith theory. The object of this discussion is summarised below:

A. to show how far the initial objects of the work have been achieved

B. to assess the position of the statolith theory in the light of this

C. to make an appraisal of the alternative theories

D. to suggest how geoperception by statoliths could trigger off geotropic response

E. to suggest how geoperception by alternative means could trigger off geotropic response

F. to suggest lines for further research

DISCUSSION

The supporters of the statolith theory rely largely on the wide distribution of statolith cells in those places which are known to be sensitive to gravity. As shown by Tischler (1905) in roots which are not geotropic statolith starch is not formed or is formed only in small amounts; in cases of temporary lack of geotropic sensitivity the movable starch appears at the same time as geotropic sensitivity. Conflicting observations were made by Linsbauer (1907) who saw statoliths in ageotropic roots of Aroids. However, such an observation does little, if anything, to detract from the statolith theory since such starch grains may well be a vestige of a potentiality for geoperception lost during the course of evolution; maybe one or more of the subsequent reactions in the chain leading to response has been lost by these roots.

Experiments involving the elimination of statolith starch from geoperceptive organs lend support to the statolith theory but allow no unambiguous conclusions to be drawn. Complete elimination of statolith starch coincides with the complete disappearance of the geotropic reaction, while in the case of partial destarching and the gradual replenishment of the starch there is a corresponding reduction in and reappearance of the capacity to react.

The experiments described in this thesis show further how close a correlation there is between starch-statoliths and geotropic response without actually proving the starch-statolith theory. It would seem that the only way of proving the theory would be to remove

the statolith apparatus without affecting anything else and this is not possible. Consequently we have to be content with a multiplicity of correlations, none of which is absolute.

It has been shown that the presentation time for roots of Vicia faba at 21±1°C is less than one minute. During this short time the gravitational stimulus has set in train an irreversible reaction or sequence of reactions which will inevitably lead to curvature. An experiment described in this thesis (page 66) shows that the rate of starch grain movement is sufficiently high to account for such rapid perception.

If it is assumed that the movement of amyloplasts through the viscous cytoplasm onto a sensitive contact area on a lateral wall triggers off the geotropic response, the rapid movement of amyloplasts in response to changes in gravitational stimulation fits in well with the basic laws of geotropism. It has been shown, for instance, that there is a precise inverse correlation between the gravitational field (g) at the presentation time (t). In fact within the limits of experimental error g x t is constant (Rutter-Pekelharing, 1910). Since the average distance moved by the starch grain is a constant determined by linear dimensions of the sensitive cell and since the terminal sedimentation value is rapidly reached and is directly proportional to the value of g applied, then the constancy of g x t is easily explained assuming that t is equal to the time for a certain minimum number of starch grains to reach the sensitive surface and assuming that the cytoplasm is completely homogeneous.

Another characteristic of perception is that a series of successive sub-threshold stimuli can be summed, and responses are evoked only when the total exposure time equals the presentation time, provided that the intervening periods of non-stimulation do not exceed the individual stimulation periods by more than ten times (Fitting, 1905). This can be explained using the starch grain theory, for when an organ is turned from the vertical to the horizontal, the starch grains fall down the formerly lower wall to the lateral wall which is now lowermost. If before they reach it they are halted by the return of the organ to the vertical, they should remain stationary for a time in that position until a return to the horizontal starts them falling again. Since the terminal velocity is rapidly reached, the distance moved is virtually proportional to the exposure time, and it follows that strict summation of the effects of each horizontal exposure would then be expected (Audus, 1962).

The results of curvature measurements made during rotation following horizontal stimulation of sufficient duration for the starch to have fallen to the lowermost walls correspond on the basis of the statolith theory with the results expected from the observed positions of the amyloplasts: the unilateral distribution of starch grains resulting from horizontal stimulation was followed by curvature during rotation, while the subsequent redistribution of starch grains caused by rotation was followed by growth in the direction of the axis of rotation (Chapter II, D).

A further experiment involving rotation which would throw light on the validity of the statolith theory could be based on the findings of Knight (1806) who rotated seedlings rapidly enough (150 r.p.m.) to produce a centrifugal force roughly 3.5 times the force of gravity. As a result of this the roots of the seedlings grew outwards from the centre of the wheel and the shoots towards the centre. If a series of experiments were to be performed to determine the minimum speed of rotation necessary to cause such curvature responses, an interesting correlation could be made with the minimum speed of rotation necessary to cause unilateral distribution of starch. It would also be valuable to repeat some of the experiments of earlier workers who obtained results which conflict with the statolith theory, to discover whether the discrepancy is due to slow fixation or whether it is real.

Further support for the statolith theory is afforded by the experiments in which the effects of low temperatures on starch movement and on the direction of growth were compared (Chapter II, E). Here again the conclusions to be drawn are not absolute. One can only say that the close similarity between the reduction in the amount of starch movement at low temperatures and the reduced degree of geotropic curvature at low temperatures lends support to the theory; it must not be forgotten that starch movement is the only other factor that has been measured besides curvature, and that other factors will be affected by low temperatures which may lead to the reduction in curvature. In the experiment the low temperature treatment was confined as far as possible to the perception process. The plants were transferred to room temperature immediately after stimulation.

Horizontal stimulation at room temperature will lead to curvature even if the stimulation time does not exceed one minute, indicating that only perception needs to occur during this period of horizontal stimulation and that subsequent stages in the reaction chain can occur after stimulation. If it were these subsequent stages that were being prevented by low temperature and not perception, one would expect that once the root was transferred to room temperature, the perception process would trigger off the subsequent reactions leading to curvature. It would seem most probable then that the reason why the roots do not curve after horizontal stimulation at temperatures low enough to prevent the movement of amyloplasts is because such temperatures are preventing the perception process. We cannot say for certain though that this perception process is the movement of the amyloplasts within the cell.

The shortness of the presentation time has been of use in considering the plausibility of the statolith theory. It is also an important characteristic to consider in evaluating any of the other theories which have been propounded to explain the ability of plant organs to perceive and respond to the influence of gravity. It can lead us on to consider the sedimentation or creaming of particles in the cytoplasm quite apart from the sedimentation of amyloplasts.

There are many organelles which have at one time or another been proposed as candidates for the function of graviperceptor. These range from the nucleus to the smallest particles such as microsomes. Microscope observations of the statolith cells show that there is no obvious movement of the nucleus under the action of gravity,

so this can be ignored as a possible graviperceptor. On the other hand, reports of mitochondrial movement have been made as a result of light microscope observations in which mitochondria have been recognised by their ability to reduce colourless tetrazolium salts to red formazan (Ziegler, 1954). More recent work on the histochemical localisation of enzyme activities indicate biochemical heterogeneity of mitochondria (Novikoff, 1957; Avers, 1961; Sedar and Rosa, 1961). It is possible that the observations of Ziegler might have represented localisation of mitochondria with succinic dehydrogenase activity, while others without remained undetected, or they might represent localisation of suitable conditions for the activity of succinic dehydrogenase. If this is the case, the observations of Ziegler represent secondary effects of gravity rather than primary ones.

Knowledge of the presentation time and calculations based on theoretical grounds can help us in assessing the validity of Ziegler's report of the sedimentation of mitochondria. By using Stokes's law the approximate rates of sedimentation of mitochondria and other organelles have been calculated (Audus, 1962). In making these calculations a cytoplasmic density of 1.0 and a viscosity of 20 centipoises were taken, corresponding to a value determined for the stem parenchyma of Vicia faba seedling by Heilbronn (1914) and based on actual observations of the rate of fall of amyloplasts. The table of the results of these calculations is given overleaf.

Particle	Diameter	Density assumed	Rate of fall $cm.sec^{-1}$	Time to travel 10 μ	Time to travel particle diameter
Starch grain	2 μ	1.5	5.5×10^{-6}	3.0 min	36 sec
Mitochondrion	0.5 μ	1.2	1.4×10^{-7}	2 hr	6 min
Ribosome	300 Å	1.2	5×10^{-10}	23 days	100 min
Oil droplet	300 Å	0.88	3×10^{-10}	38 days	170 min
Protein molecule	50 Å	1.35	2.4×10^{-11}	47.5days	5 hr

From these figures it is possible to calculate the distance each of these particles would travel during the presentation time. The presentation time has been calculated for Vicia faba by three different methods. These have led to values of approximately 0.3, 0.6 and 1.0 minutes. The estimation of the distance which particles would move in the presentation was made using the largest estimate (i.e. one minute) of the presentation time.

Organelle	Distance which organelle would move in one minute
Starch grain	3.3 μ
Mitochondrion	8.4×10^{-2} μ
Ribosome	3 Angstrom
Oil droplet	1.8 Angstrom
Protein molecule	1.44 Angstrom

From this one can conclude that if any degree of accumulation of particles on one side of the cell is required for triggering off the geotropic reaction, then falling starch grains could account for even the shortest presentation time, even allowing that the density of cytoplasm may be appreciably greater than 1.0, which seems unlikely since the observed rate of starch sedimentation is as fast or faster than the calculated rate. None of the other particles moves sufficiently quickly to account for the short presentation time if, as suggested is implied by the results of the rotation experiments on page 79, accumulation of some of the perceptor particle on one side of the cell is necessary.

If only a slight particle displacement is necessary, for example by an amount just necessary to bring a mitochondrion into contact with some internal membrane, then, since the particle could move through 8.4×10^{-2} μ (according to these calculations) during the presentation time it is just conceivable that the movement of mitochondria could lead to geoperception.

The calculated rates of sedimentation of particles of microsomal dimensions are so small as to make these particles extremely unlikely graviperceptors. In fact, the tendency for such particles to settle under the influence of gravity would be opposed by thermal agitation and by particle flux caused by diffusion due to the concentration gradient set up by the sedimentation, so that sedimentation would be even slower than calculated here.

In contrast with the theoretical considerations of Professor L.J. Audus presented on the previous page, which lead to the conclusion that amyloplasts and possibly mitochondria or dictyosomes are the only particles which could move sufficiently quickly to perceive changes in the gravitational field and cause response by curvature, Professor H. Hertz (1962, private communication) has calculated the theoretical particle size of particles which he believes would move sufficiently quickly to explain the shortness of the presentation time and found it to be around 1μ. Unlike Professor Audus, Professor Hertz believes that particles of the size of mitochondria would sediment in about 20 minutes to the lowermost sides of the cells. The variation in theoretical values arise partly from the great difference in the assumed viscosity of the cytoplasm by the two authors. Professor Audus works on the assumption that the viscosity of cytoplasm is of the order of 20 centipoises. This assumption is based on observations of the rate of fall of amyloplasts in stem parenchyma cells of Vicia faba (Heilbronn, 1914). Hertz bases his calculations on the assumption that the viscosity of the cytoplasm is about 10 centipoises. If one considers Stokes's law which gives the rate of fall (V) of a small sphere in a viscous fluid by the equation

$$V = \frac{2ga^2 (d_1 - d_2)}{9\eta}$$

where a is the radius of the sphere, d, and d_2 are the densities of the sphere and the medium respectively, V is the rate at which the sphere moves under the influence of gravity g, and η is the coefficient of viscosity, it is evident that the value which Professor Audus estimates for V will be half

as great as Professor Hertz's estimate.

Since this difference only partly accounts for the discrepancy in the theoretical calculations of Hertz and Audus, the rest must be due to their estimates of the densities of the sphere or of the medium.

The shortness of the presentation time leads to serious criticisms of the recent theory proposed by Larsen (1959, 1961) involving submicroscopic particles. He suggests that the graviperceptors behave like hinged particles of macromolecular dimensions or larger which swing like pendulums under the influence of gravity, their final orientation depending partly on the direction of the gravitational field and partly on the electrostatic fields in the cell and possibly also on the anisotropy of the cytoplasm. If these pendulums were mitochondria, their rate of orientation under gravity would possibly be sufficient to account for the observed presentation time, but any smaller particles would be far too slow. It is difficult to visualise a method for detecting re-orientation of particles, even with the aid of an electron-microscope, unless the microvilli of the mitochondria take up a particular orientation with respect to the direction of the gravitational field. Even so, since the microvilli are radially arranged at frequent intervals around the mitochondria, changes in orientation would be extremely hard to detect.

In conclusion then, it would seem that the only particles capable of sufficiently fast sedimentation to cause response to changes in the orientation of a plant organ are starch grains, mitochondria and dictyosomes. If any of these is the graviperceptor, how is its distribution in the cell to be linked to the response?

Presumably since the mitochondrion is such an important component of the metabolic system, the unequal distribution of mitochondria would cause metabolic gradients laterally across the cell, thus modifying the cell behaviour. But since it seems doubtful on theoretical grounds that mitochondria could become unilaterally distributed during the presentation time, we must think along other lines. Possibly their contact with the upper surfaces of cytoplasmic membranes in the cell leads to the development of physiological gradients in the cell which would lead to response. Or possibly they lead to the development of potential differences in the cell as suggested by Hertz. However, it is difficult to visualise how such randomly situated effects could possibly be co-ordinated to lead to unilateral auxin concentration and response by curvature.

If the graviperceptors are metabolically inactive particles which accumulate on one side of the cell as the starch grains might be, they could modify the activity of stationary metabolic units by direct contact, or lower their effectiveness by competing for their space and forcing them to some other part of the cell. This again would give rise to metabolic gradients in the perception cell which could lead to curvature.

The movement of one group of particles may indirectly affect the distribution of others. Particles may adhere, the heavier carrying the lighter with them as they sediment. Theoretical considerations cannot lead us to the answer concerning the truth of this. The electron microscope probably can. It can also show us how far the organelles sediment under the direct action of gravity. For example, the discrepancies

arising from theoretical considerations should be solved by direct observation.

However, even direct observation has failed to lead to a definite answer: Ziegler, by the use of tetrazolium salts and the light microscope has clearly demonstrated unilateral reduction of the tetrazolium salt and he believes this to be indicative of the unilateral distribution of mitochondria and of their rapid sedimentation under the influence of gravity. By the use of the electron microscope on the other hand, I have been unable to demonstrate anything but a random distribution of mitochondria in the statolith cells. Roots which did show differences in the numbers of mitochondria in the four sectors, had less mitochondria in the lower sectors.

Dictyosomes are less in number in the lowermost distal sector than in the two uppermost sectors. It has been suggested (Mollenhauer, Whaley and Leech, 1961; Drawert and Mix, 1962 and Sievers, 1963) that dictyosomes are concerned with cell-wall growth. They appear to produce large vesicles containing electron-dense substance. The vesicles appear to pass through the cytoplasm and through the plasma membrane. There is a close packing of these bodies between the surface of the protoplast and the surface of the cell wall. Ultimately they disappear, the cell wall volume increases and the protoplast returns to its original smooth contour. If the higher dictyosome concentration in the upper halves of the cells of the root-cap is to be considered as an important part of the geoperception process it is necessary to use the postulation of Professor Audus (1962) that the sensitive cells have a radial polarity. This is

discussed in the introduction (pages 30-33). Without such polarity, the co-ordination of the growth response of the root as a whole would not be possible, and the cells would be under considerable strains caused by inequalities in growth of individual cells in the tissue. Any redistribution of anything inside a cell cannot of itself lead to differences on the two sides of the organ. These must be associated with some kind of radial polarity in the organ such that the outer sides of the cells are (structurally or biochemically or both) different from the inner sides of the cell.

The observation that there tends to be more endoplasmic reticulum in the upper halves of the root-cap cells of roots which have been horizontal is interesting in relation to the remarkable observations of Nemec (1900, 1901) made during his survey of statocytes in plants. The densely staining protoplasmic bodies of lamellate and granular nature which he observed in horizontal organs at the ends of the cell where starch grains are normally situated appeared when the starch grains moved from those walls under gravity and were pushed out of position by returning starch grains on placing the organ vertical again. They then slowly disappeared. It was this interaction of starch statolith with protoplasm which Nemec regarded as initiating the reaction sequence leading to curvature. Possibly Nemec's lamellate protoplasmic bodies are analogous to the aggregation of endoplasmic reticulum which was often observed opposite the starch grains in horizontal roots.

Mollenhauer (private communication, 1963) has suggested that the masses of endoplasmic reticulum observed in stimulated roots are perhaps artefacts of fixation. He has observed whirls of endoplasmic

reticulum in outer root-cap cells of maize. These whirls however are very different from the parallel membranes observed in the inner root-cap cells and are probably not comparable. The endoplasmic reticulum masses recorded in this thesis are regular unbroken parallel membranes in cells which do not give other indications of damage by fixation.

The variance ratio for all the roots taken as a whole indicates that there is no significant difference between the amount of endoplasmic reticulum in the four sectors of the root. However, the variance ratio of the endoplasmic reticulum results must be decreased considerably by the nature of the method employed: the majority of the endoplasmic reticulum is around the periphery of the cell. Therefore truly median sections cut exactly at right angles to the horizontal plane and along the long axis of the root would be the only ones which would give truly comparable results for the measurements between sectors. But such a measurement probably did not occur among the 70 cells. One can hope though that the choice of cells was sufficiently random to eliminate any bias in one direction or the other, and that inaccuracies in cutting in the right plane and the inability to detect a truly median section would mask the true effects of gravity on cellular organization rather than create new ones. In view of these considerations it is worth considering the tendency for there to be less endoplasmic reticulum in sectors A and D, although the overall values do not attain statistical significance at the 0.05 probability level.

Indications that the endoplasmic reticulum is connected with wall growth in root-tip cells have been observed by Dr. B.E. Juniper (private communication, 1963). He has observed a close correlation throughout the root-cap of the amount of endoplasmic reticulum and the amount of wall-growth. There is virtually no endoplasmic reticulum in the quiescent centre of the root-tip of maize. Such endoplasmic reticulum as there is, is scattered and unorientated in the cell. There is no wall building there. There is also little endoplasmic reticulum in the meristematic cells of the root tip of maize - e.g. 100 μ behind the root-cap junction. This is the site of the most rapid cell division but very little wall-making as such. Such endoplasmic reticulum as there is, is beginning to be orientated parallel to the cell walls. The major zones of wall synthesis are the differentiating cells of the elongating zones of the root about 3 mm. behind the root cap junction and the rapidly elongating and maturing cells of the cap beyond 60 μ from the cap-junction where division has ceased. In the cortical cells of the root similar indications of a connection between endoplasmic reticulum and active wall synthesis occur. In the cortex thick walls occur between cell and intercellular space and thin walls on cell to cell contacts. Multiple sheets of endoplasmic reticulum are present parallel to the thick walls and little if any parallel to the thin. Such as there is at the thin walls may be at right angles to the wall. The cap initials of maize have orientated endoplasmic reticulum parallel to the distal walls, i.e. those which will be experiencing rapid growth, and little if any parallel to the cap junction wall which will not be growing. This is regardless of the orientation of the root as a whole.

In the cap cells it seems that the ER can be re-orientated depending on the orientation of the whole root. In roots which are grown in a normal vertical position the ER is concentrated to a certain extent at the proximal end of the cell. However this is not absolute, although it is most probably significant.

With this suggested direct function of endoplasmic reticulum in wall-growth in mind, it is difficult to use it to correlate redistribution in the cap-cells as a result of a change in gravitational stimulation with the growth response resulting from such a change, since the response is far removed from the cap cells. One could suggest that endoplasmic reticulum is the site of synthesis of indole acetic acid and that the precursor of indole acetic acid, or the enzymes involved in its conversion to indole acetic acid is localised on the endoplasmic reticulum membranes, while either the precursor or the enzymes (whichever is not on the membranes) is situated in the cytoplasm of the inner sides of the cell, so that horizontal stimulation will bring the precursor and the enzymes for converting the precursor to indole acetic acid together only in the upper halves of the cells below the central horizontal axis. Such behaviour would explain the increase in the amount of auxin produced in the lower half of the root during horizontal stimulation. A similar function could be postulated for the dictyosomes. However, before speculating further on the significance of the redistribution of the endoplasmic reticulum and dictyosomes in relation to graviperception it is important to make a wide survey of starch statocytes in different plants

and organs to check whether it is a general phenomenon. It is important also to find an accurate method for detecting and recording endoplasmic reticulum distribution which would be more susceptible to statistical analysis.

Summary of Electron Microscope Observations.

The only modification of cell ultrastructure observed in horizontally stimulated roots was the electron-dense layer which was seen at the lowermost cell-wall/cytoplasm boundaries. This was shown to be a fixation artefact rather than an effect of gravity.

Besides the increase in the number of plastids at the lower sides of the cells after horizontal stimulation which has been long since known, slight movement of dictyosomes has been observed. This movement is towards the upper side of the cell and may result from displacement of the cytoplasm by plastids. A similar tendency is observed in the distribution of mitochondria, but although this attains statistical significance in some of the roots, the difference between the means of all the roots is not large enough to be regarded as statistically significant.

The endoplasmic reticulum shows a tendency to accumulate in the upper half of the cell, but after 20 minutes of horizontal stimulation this tendency is not sufficiently general as to be regarded seriously as an important feature in geoperception.

CONCLUSIONS

1. The amyloplast ("starch") statolith theory still remains unproven, but the evidence in its favour is strong. The mechanism whereby gravity can affect plant cells must be either by bringing about displacements or re-orientation of cell components by virtue of density differences between the moving component and its environment.

2. On theoretical grounds nothing smaller than mitochondria could change position rapidly enough, even if the distance moved by the particle sufficient to lead to geoperception were commensurate with the size of the moving particle.

3. On experimental evidence including electron microscope observations, the starch grains within the plastids of the geoperceptive cells is still the strongest candidate for the rôle of graviperceptor in the root-tip. Electron microscope studies have indicated ways in which the sedimentation of amyloplasts of root-tip modifies the internal organization of the sensitive cell.

4. The geoelectric effect is probably a secondary effect of gravitational stimulation which may or may not be an integral part of the reaction sequence leading to geotropic curvature. Recent experiments (Hertz, private communication, 1963) indicate that the geoelectric effect is closely bound to the transverse movement of auxin and that if there is no auxin there is no geoelectric effect. The geoelectric effect is in all probability a metabolic effect and is almost certainly the indirect result of graviperception.

5. Statolith sedimentation is therefore considered as the most likely mechanism which triggers off the sequence of reactions leading to curvature. This may be by modifying the internal organisation of the sensitive cell either by changing the spatial distribution of other cell components or by changing the biochemical activity of some cell components or regions.

6. Changes in mitochondrial distribution resulting from displacement by the sedimented amyloplasts are not considered as general enough to be considered an important part of the reaction sequence.

7. Dictyosomes are more numerous in the upper halves of the cells after the roots have been horizontal for 20 minutes. The difference is small between upper and lower halves of the cells. It probably results from displacement of the cytoplasm containing dictyosomes and mitochondria. There is also a tendency for there to be more mitochondria in the upper halves of the cells. These changes are not regarded as sufficiently large to be considered as an important part of the perception process.

8. The endoplasmic reticulum is in general rather more highly concentrated in the upper halves of the root-cap cells. The possible function of endoplasmic reticulum in geotropic response is considered in relation to the observations of Němec (1900, 1901).

9. Speculations have been entertained concerning the possible role of the dictyosomes and the endoplasmic reticulum in inducing the differential production

or release of a growth substance. For these, radial polarity of the roots has been assumed.

10. The final conclusion is that the results of this work indicate that it is the movement of the amyloplasts *per se* that provide the primary stimulus, *not* the associated movement of other organelles or the direct sedimentation or creaming of those organelles *per se*.

A P P E N D I X

MITOCHONDRIA PER SECTOR

Transformed figures. - (formula used $\sqrt{x}$)

Root	No. of cells	Sector A	Sector B	Sector C	Sector D	Mean
I	14	3.11	3.04	3.10	3.04	3.07
II	4	3.58	2.83	2.75	3.31	3.12
III	6	3.46	3.55	3.89	3.66	3.64
IV	3	3.87	3.51	3.62	3.57	3.64
V	3	3.88	3.51	3.00	2.97	3.34
VI	4	3.54	3.98	3.56	3.64	3.68
VII	7	3.84	2.96	3.94	3.05	3.45
VIII	8	2.75	3.97	3.30	2.91	3.23
IX	8	2.99	3.41	2.84	3.00	3.06
X	8	3.44	3.80	3.08	2.82	3.28
XI	5	3.24	2.72	1.97	2.68	2.65
MEAN		3.32	3.37	3.19	3.09	3.24

GOLGI BODIES PER SECTOR

Transformed figures - (formula used $\sin^{-1}\sqrt{\frac{x}{X}}$
where X = 21 = greatest number observed)

Root	Sector A	Sector B	Sector C	Sector D	Mean
I	37.95	31.72	28.85	33.10	32.90
II	33.02	34.20	37.59	37.25	35.51
III	29.77	36.18	38.87	32.42	34.31
IV	23.17	36.92	42.29	29.07	32.86
V	39.88	30.43	37.41	28.43	34.04
VI	38.50	43.62	40.85	35.53	39.63
VII	34.34	40.07	38.69	30.49	35.90
VIII	31.38	39.74	37.74	33.81	35.67
IX	37.74	28.14	21.62	28.26	28.94
X	37.55	41.15	31.27	18.85	32.20
XI	34.04	29.15	19.77	23.16	26.53
MEAN	34.99	35.33	32.64	29.97	33.23

Area of Sector occupied by E.R. (mg)

Untransformed figures

Root	L.S.D.	Sector A	Sector B	Sector C	Sector D	Mean
I	27.6	27.9	39.0	41.1	6.1	28.6
II	53.7	54.0	35.8	61.0	4.5	38.8
III	43.7	52.7	45.7	24.8	41.3	41.1
IV	63.2	0	31.0	67.3	0	24.6
V	63.2	24.3	60.3	4.7	7.7	24.3
VI	53.7	42.5	39.8	29.0	22.5	33.4
VII	40.5	27.3	71.1	16.9	28.9	36.0
VIII	37.8	54.3	30.0	9.5	21.8	28.9
IX	37.8	42.3	45.6	73.8	29.8	47.8
X	37.8	28.0	8.5	18.3	46.3	25.3
XI	49.6	0	33.4	73.2	6.8	28.4
MEAN		33.61	39.06	37.10	21.19	

Least significant difference (L.S.D.) =

$$\frac{\text{Residual standard deviation}}{\sqrt{n}} \times \sqrt{2} \times t$$

Golgi Bodies per 100 mg of Free Cytoplasm.

Untransformed figures

Root	L.S.D	Sector A	Sector B	Sector C	Sector D	Mean
I	1.48	4.672	3.421	3.064	3.905	3.766
II	2.80	2.865	4.291	4.782	4.150	4.022
III	2.28	3.343	3.475	4.586	4.430	3.958
IV	3.24	1.636	3.492	3.971	3.210	3.077
V	3.24	4.374	2.873	5.191	2.741	3.795
VI	2.80	3.325	4.054	4.552	3.585	3.879
VII	2.10	3.279	3.669	4.267	3.814	3.757
VIII	1.96	3.143	3.740	5.183	4.610	4.169
IX	1.96	4.477	2.965	2.322	3.764	3.382
X	1.96	4.761	5.969	5.072	2.239	4.510
XI	2.49	5.543	4.457	2.230	4.348	4.144
MEAN		3.971	3.865	3.955	3.763	

Mitochondria per 100 mg Free Cytoplasm

Untransformed figures

Root	L.S.D	Sector A	Sector B	Sector C	Sector D	Mean
I	2.45	5.2131	5.3440	6.2799	6.5197	5.8392
II	4.59	5.8590	5.3473	4.5980	5.6453	5.3524
III	3.75	8.9122	6.4272	8.6453	9.8280	8.4532
IV	5.31	7.9333	6.6297	5.8453	7.4927	6.9753
V	5.31	8.1530	5.9657	6.3677	5.0787	6.3913
VI	4.59	6.1528	6.1080	6.5228	6.4018	6.2963
VII	3.48	7.3400	3.7699	8.0051	6.0534	6.2921
VIII	3.24	4.2859	7.9099	7.2399	6.3104	6.4365
IX	3.24	5.2615	6.9865	6.3111	6.8500	6.3523
X	3.24	7.0949	9.5426	7.4695	6.8640	7.7428
XI	4.13	8.9466	6.2320	3.5758	13.7612	8.1289
MEAN		6.4573	6.4292	6.6142	7.2503	

E.R. per 100 mg Free Cytoplasm

Untransformed

Root	L.S.D	Sector A	Sector B	Sector C	Sector D	Mean
I	15.9	15.115	20.547	25.711	3.322	16.170
II	29.7	26.694	20.106	29.157	2.586	19.636
III	24.3	27.184	16.303	9.477	17.265	17.557
IV	34.5	0	11.047	32.715	0	10.941
V	34.5	14.006	29.575	2.333	5.476	12.848
VI	29.7	14.828	13.731	11.702	15.046	13.827
VII	22.6	12.524	32.154	7.606	16.737	17.255
VIII	21.0	34.337	17.097	5.398	15.925	18.189
IX	21.0	19.222	26.725	47.294	18.328	27.892
X	21.0	17.825	4.398	16.007	40.740	19.742
XI	26.7	0	25.692	54.428	12.009	23.032
MEAN		17.737	19.743	22.291	14.489	18.565

ANALYSIS OF VARIANCE TABLES

Mitochondria (using transformed figures)

Source of Variance	Sum of Squares	d.f.*	Mean Square	F. Ratio
Between Roots	20.01	10	2.00	
Between Sectors	3.41	3	1.14	
Cells within Roots	53.70	59	.91	
Root x Sector	24.09	30	.80	1.2 N.S.
Residual	118.49	177	.67	
TOTAL	219.70	279		

Adding back non-significant interaction

Between Roots	20.01	10	2.00	2.20 20/1
Between Sectors	3.41	3	1.14	1.65 not sig.
Cells within Roots	53.70	59	.91	1.3 = 20/1
Residual	142.57	207	.69	
TOTAL	219.70	279		

Residual S.D. = .688763 = .8299

S.E. of cells within roots = $\frac{.91 - .69}{4}$ = .24

S.E. between sectors is assumed not to differ significantly from 0

S.E. between roots = $\frac{2.00 - .91}{4 \times 6.36}$ = .21

* d.f. = degrees of freedom

ANALYSIS OF VARIANCE TABLES

Golgi bodies (using transformed figures)

Source of Variance	Sum of Squares	d.f.*	Mean Square	F. Ratio
Between Roots	2,691.32	10	269.13	
Between Sectors	1,290.67	3	430.22	
Cells within roots	12,442.32	59	210.89	
Root x Sector	5,423.34	30	180.78	1.37 N.S.
Residual	25,413.24	177	132.28	
TOTAL	45,260.89	279		

Adding back non-significant interaction

Roots	2,691.32	10	269.13	1.28 N.S.
Sectors	1,290.67	3	430.22	3.09 20/1
Cells within Roots	12,442.32	59	210.89	1.51 = 20/1
Residual	28,836.58	207	139.31	
TOTAL	45,260.89	279		

Residual S.D. = $\sqrt{139.31}$ = 11.80

Cells within roots S.E. = $\frac{210.89 - 139.31}{4}$ = 4.23

S.E. between sectors = $\frac{430.22 - 139.31}{11 \times 6.36}$ = 2.04

S.E. between roots is assumed not to differ significantly from 0.

* d.f. = degrees of freedom.

ANALYSIS OF VARIANCE TABLES

Area of Sector occupied by E.R.

Source of Variance	Sum of Squares	d.f.*	Mean Square	F. Ratio
Between Roots	15,177.78	10	1,517.78	
Between Sectors	13,524.49	3	4,508.16	
Cells within roots	114,401.71	59	1,939.01	1.36 N.S.
Root x Sector	75,276.15	30	2,509.21	1.75 20/1
Residual	253,187.36	177	1,430.44	
TOTAL	471,567.49	279		

Adding back non-significant term

Between Roots	15,177.78	10	1,517.78	1 not sig.
Between Sectors	13,524.49	3	4,508.16	1.80 not sig.
Root x Sector	75,276.15	30	2,509.21	1.61 20/1
Residual	367,589.07	236	1,557.58	
TOTAL	471,567.49	279		

Residual S.D. = $\sqrt{1,557.58}$ = 39.47

Root x Sector Interaction S.E. = $\frac{2,509.21 - 1,557.58}{6.36}$ = 12.23

S.E. between sectors is assumed not to differ significantly from 0.

S.E. between roots is assumed not to differ significantly from 0.

* d.f. = degrees of freedom.

ANALYSIS OF VARIANCE TABLES

Golgi bodies per 100 mg free cytoplasm

Source of Variance	Sum of Squares	d.f.*	Mean Square	F. Ratio
Between Roots	34.15	10	3.41	
Between Sectors	1.93	3	.64	
Cells within roots	291.93	59	4.95	1.36 N.S.
Root x Sector	193.67	30	6.46	1.77 100/1
Residual	644.50	177	3.64	
TOTAL	1166.18	279		

Adding back non-significant term

Between Roots	34.15	10	3.41	1 not sig.
Between Sectors	1.93	3	.64	1 not sig.
Root x Sector	193.67	30	6.46	1.63 100/1
Residual	936.44	236	3.97	
TOTAL	1166.18	279		

Residual S.D. = 3.9679 = 1.99

Root x Sector interaction S.E. = $\frac{6.46 - 3.97}{6.36}$ = .63

S.E. between sectors is assumed not to differ significantly from 0.

S.E. between roots is assumed not to differ significantly from 0.

* d.f. = degrees of freedom.

ANALYSIS OF VARIANCE TABLES

Mitochondria per 100 mg free cytoplasm

Source of Variance	Sum of Squares	d.f.*	Mean Square	F. Ratio
Between Roots	234.89	10	23.49	1.43 not sig.
Between Sectors	30.93	3	10.31	1 not sig.
Cells within roots	970.68	59	16.45	1.53 20/1
Root x Sector	518.74	30	17.29	1.61 20/1
Residual	1,899.31	177	10.73	
TOTAL	3,654.55	279		

Residual S.D. = $10.7306 = 3.28$

Root x Sector Interaction S.E. = $\frac{17.29 - 10.73}{6.36} = 1.02$

Cells within roots S.E. = $\frac{16.45 - 10.73}{4} = 1.20$

S.E. between sectors is assumed not to differ significantly from 0.

S.E. between roots is assumed not to differ significantly from 0.

* d.f. = degrees of freedom.

ANALYSIS OF VARIANCE TABLES

E.R. per 100 mg free cytoplasm

Source of Variance	Sum of Squares	d.f.*	Mean Square	F. Ratio
Between Roots	5,092.02	10	509.20	
Between Sectors	2,280.20	3	760.07	
Cells within roots	30,749.73	59	521.18	1.21 N.S.
Root x Sector	31,719.61	30	1057.32	2.46 1000/1
Residual	75,965.68	177	429.18	
TOTAL	145,807.24	279		

Adding back non-significant term

Between Roots	5,092.02	10	509.20	1.13 N.S.
Between Sectors	2,280.20	3	760.07	1 not sig.
Root x Sector	31,719.61	30	1057.32	2.34 1000/1
Residual	106,715.42	236	452.18	
TOTAL	145,807.24	279		

Residual S.D. = $\sqrt{452.1840}$ = 21.26

S.E. Root x Sector Interaction = $\frac{1057.32 - 452.18}{6.36}$ = 9.75

S.E. between sectors is assumed not to differ significantly from 0.

S.E. between roots is assumed not to differ significantly from 0.

* d.f. = degrees of freedom.

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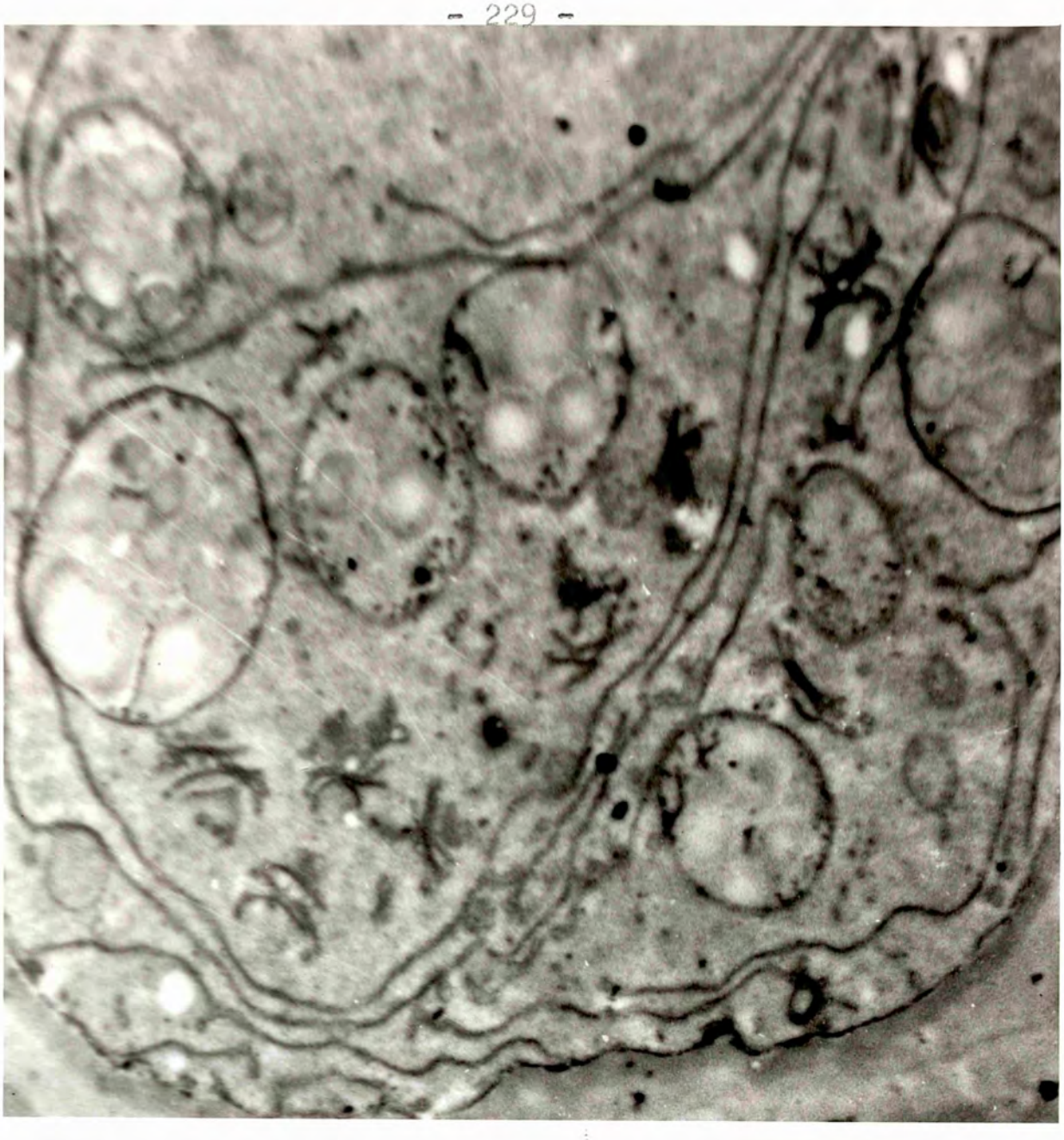
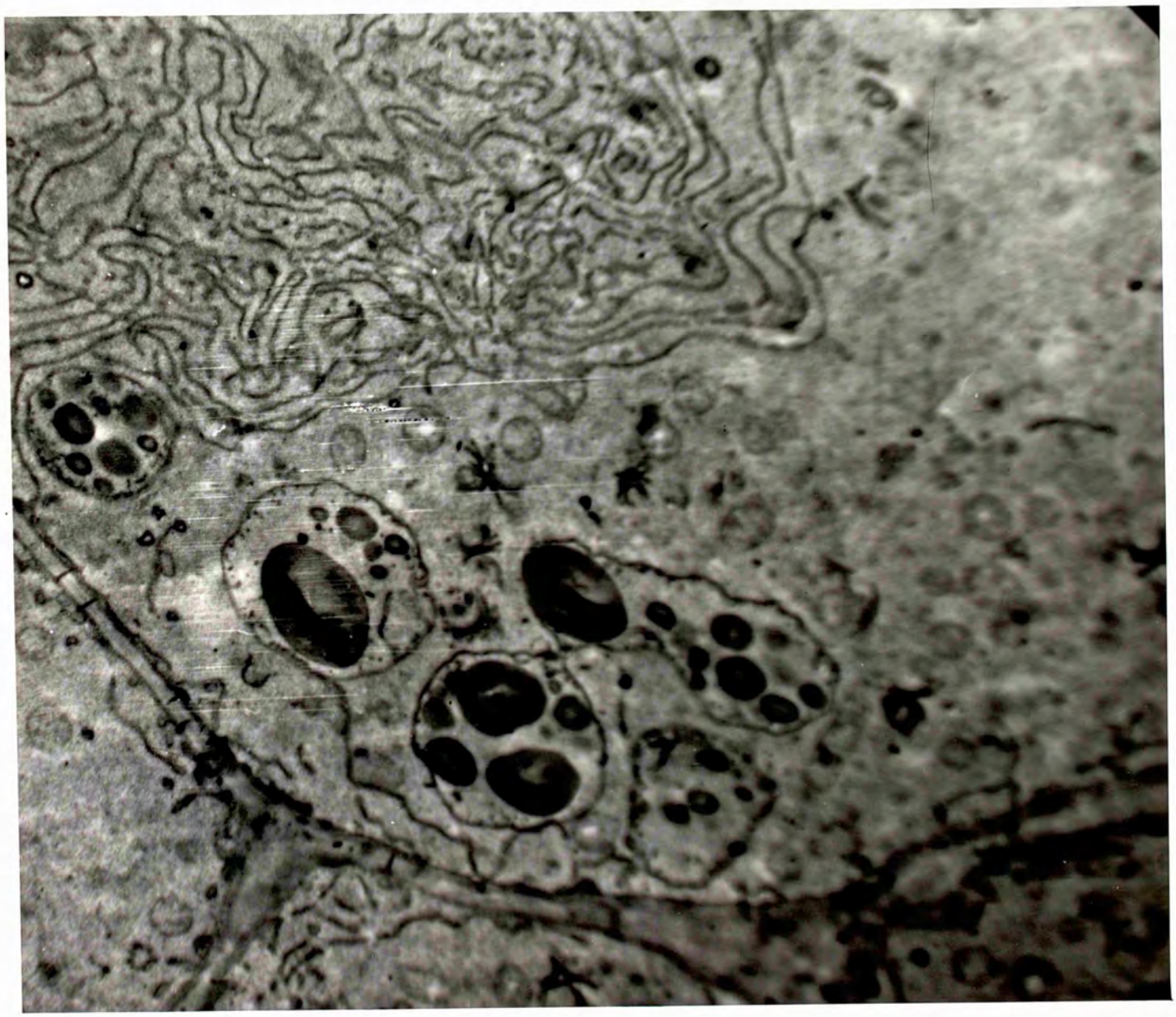
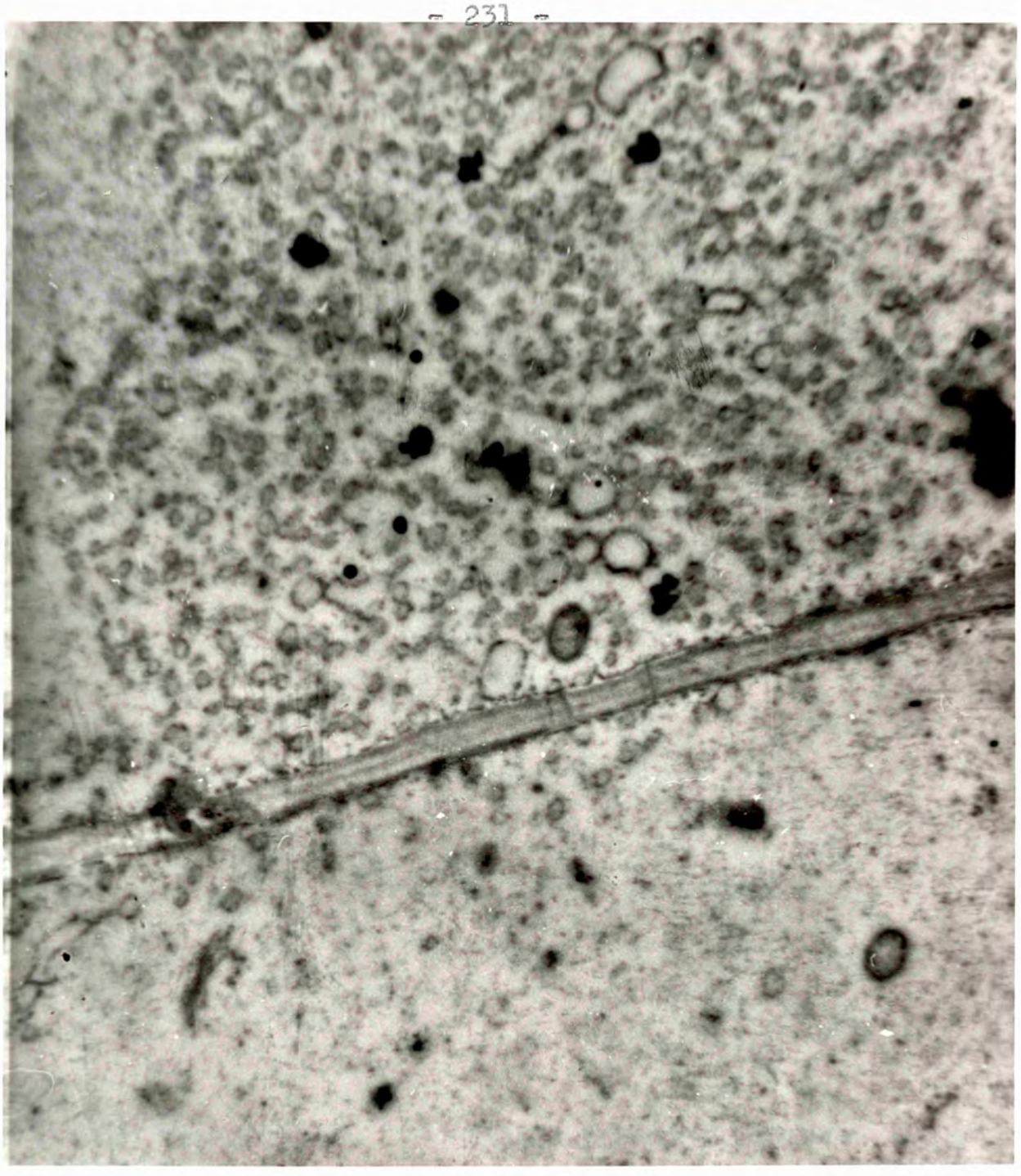


Unstimulated root-cap cell. X 5,000. $KMnO_4$. Siemens.

Root-cap cell from root which had been horizontal for 15 minutes. $KMnO_4$. X5, 000. Siemens.





Stimulated root-cap cell fixed with OsO_4. Note the difference between the cytoplasm below and above the cell wall. X10,000. Siemens.